GENETIC FUNCTIONS REQUIRED FOR GENE SILENCING IN MAIZE

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RELATED APPLICATION

[0002] This application claims the benefit under 35 U.S.C. §119(e) of United States provisional patent application number 60/238,137 filed October 5, 2000 which is hereby incorporated by reference in its entirety.

FIELD OF THE INVENTION

[0003] This invention is in the field of plant breeding. In particular, this invention relates to the isolation of new maize mutants with reduced gene silencing activity.

BACKGROUND OF THE INVENTION

[0004] The goal of *Zea mays L.* (corn) breeding is to combine various desirable traits in a single variety/hybrid. Such desirable traits include greater yield, better stalks, better roots, resistance to pesticides, pests and disease, tolerance to heat and drought, reduced time to crop maturity, better agronomic quality, higher nutritional value, and uniformity in germination times, stand establishment, growth rate, maturity and fruit size.

[0005] Modern molecular biology and transgenic technologies (genetic engineering) have greatly accelerated the introduction of new genes and, hence, new traits into corn lines. While useful, genetic engineering is hampered by transgene silencing problems. Transgene silencing is a little understood process by which genes introduced by genetic engineering are silenced or turned off. As such, transgene silencing is a major impediment to the use of genetic engineering for corn improvement. At present, the solution to transgene silencing is to search through a large number of transgenic events for transgene loci that are active and stable. This is a painstaking and laborious process, which greatly increases the cost of corn breeding using genetic engineering techniques.

[0006] In addition to transgene silencing, there are other examples of gene silencing that are variable, unstable, but heritable. In corn these include the cycling of transposable elements between active and inactive states and paramutation, gene silencing that occurs through interactions between specific alleles of a gene. The mechanism of silencing is not understood in any case, but current hypotheses invoke heritable alterations to chromatin structure.

[0007] There are no known mutants in corn that can prevent or reverse gene silencing. The availability of genetic stocks that prevent the establishment or maintenance of transgene silencing would be extremely useful for engineering and breeding new corn lines.

SUMMARY OF THE INVENTION

[0008] In order to meet these needs, the present invention is directed to new corn lines.

[0009] In particular, the present invention is directed to a mutant corn plant wherein upon propagation a change in gene activity of a paramutable allele of the mutant corn plant due to its exposure to a paramutagenic allele is reduced as compared to a wild type plant or wherein the gene activity of a paramutagenic allele of the mutant plant is not maintained as compared to a wild type plant. In one format the change in gene activity of an *B-I* allele due to exposure to a *B'* allele is prevented in the mutants. In another format, the change in gene activity of an *Pl-Rh* allele due to its exposure to a Pl' allele is reduced in the mutants. In another format, the change in activity of an *R-d* allele due to exposure to an *Pl'* allele is increased in the mutants.

[0010] The present invention is further directed to a mutant corn plant derived from a wild type corn plant wherein the mutant corn plant comprises one or more mutations that reduce the establishment, or the maintenance of paramutation or both, in the mutant corn plant as compared to the wild type corn plant. In one embodiment, the mutation is a dominant mutation. In another embodiment, the mutation is a recessive mutation.

[0011] In one format, the mutant corn plant of the invention is reduced in the establishment of paramutation at the *b1* locus. In another format, the mutant corn plant of the invention is reduced in the establishment of paramutation at the *r1* locus. In another format, the mutant corn plant of the invention is reduced in the establishment of paramutation at the *pl1* locus. In yet another format paramutation is not established at the *b1*, *r1* and/or *pl1* loci in the mutant corn plant of the invention.

[0012] In another format, the mutant corn plant is reduced in the maintenance of paramutation at the *pl1* locus. In another format, the mutant corn plant is reduced in the heritable maintenance of paramutation at the *pl1* locus.

[0013] The present invention is further directed to seed produced by the mutant corn plants of the invention. The invention is further directed to progeny seed produced by crossing the mutant corn plants of the invention with another corn plant.

[0014] The present invention is further directed to tissue culture of regenerable cells of the mutant corn plants of the invention.

[0015] The present invention is further directed to a mutant corn plant that fails to maintain the *B'* paramutant state, manifest as an increase of 2-3 fold or greater of *b1* RNA levels relative to a corresponding wild type plant and to progeny seed produced therefrom.

[0016] The present invention is further directed to a mutant corn plant that fails to maintain the *Pl'* paramutant state, manifest as an increase of 2-3 fold or greater of *pl1* RNA levels relative to a corresponding wild type plant and to progeny seed produced therefrom.

[0017] The present invention is further directed to a mutant corn plant that fails to heritably maintain the *Pl'* paramutant state, manifest as transmission of nonparamutagenic *pl1* alleles from a homozygous *Pl'* genotype, and to progeny seed and plants derived therefrom.

[0018] The present invention is further directed to a mutant corn plant, in which the normally invariant occurrence of paramutation at the *b1*, *pl1* or *r1* loci is prevented, and to progeny seed and plants derived therefrom.

[0019] The present invention is further directed to a mutant corn plant exhibiting reduced methylation of *Mutator* elements in the absence of active *MuDR* elements, relative to a corresponding wild type plant with methylated *Mutator* elements and to progeny seed and plants derived therefrom.

[0020] The present invention is further directed to a mutant corn plant wherein the mutation causes at least a two-three fold increase in transgene RNA relative to the transgene in sibling or parental wild type plants and to seed and progeny plants derived from the mutant corn plant.

[0021] The present invention is further directed to a process of producing a transgenic corn plant with an activated transgene comprising crossing a parental transgenic plant with a mutant plant wherein the parental transgenic plant has a transgene and the mutant plant is derived from a wild type corn plant. In the method, the mutant corn plant has one or more mutations that reduce the establishment or maintenance of paramutation in the mutant plant. In one format, the transgene in the parental transgenic plant is silenced and the mutation activates the silenced transgene. In another format, the transgene in the parental transgenic plant is activated and the mutation serves to prevent the transgene from being silenced in subsequent generations.

[0022] In the method of the invention a transgenic plant is produced where a silenced transgene is activated by the presence of a mutation or maintained active where the transgene is active in the parental line. The mutation reduces the establishment or maintenance of paramutation as compared to the wild type plant. In the method of the invention, the first progeny transgenic plant is outcrossed to a wild type corn plant to produce a second progeny transgenic plant wherein the silenced transgene remains activated in the second progeny transgenic plant but the second progeny transgenic plant is no longer reduced in the establishment or maintenance of paramutation as compared to the wild type plant because the mutation that originally activated the transgene is no longer present. This applies to progeny seed and plants derived therefrom.

[0023] The invention is further directed to plants produced by the methods of the invention and seed derived therefrom.

[0024] In particular, the present invention is directed to new corn plants designated *Mop1-1*; *Mop1-2EMS*; *Mop2-1*; *mop3-1*; *CC2343*; *rmr1-1*; *rmr1-2*; *rmr2-1*; *rmr7-1*; *rmr7-2*; *rmr6-1*; *rmr8-1*; *rmr9-1*; *Mop1-4*; *rmr11-1*; *Mop1-5* and to seeds derived therefrom.

[0025] In particular, the present invention is directed to corn seed designated *Mop1-1* and having ATCC Accession No. _____; corn seed designated *Mop2-1* and having ATCC Accession No. _____ ; corn seed designated *mop3-1* and having ATCC Accession No. _____ ; corn seed designated *Mop1-2EMS* and having ATCC Accession No. _____; corn seed designated *rmr1-1* and having ATCC Accession No. _____; corn seed designated *rmr1-2* and having ATCC Accession No. _____; corn seed designated *rmr2-1* and having ATCC Accession No. _____; corn seed designated *rmr7-1* and having ATCC Accession No. _____; corn seed designated *rmr6-1* and having ATCC Accession No. _____; corn seed designated *rmr8-1* and having ATCC Accession No. _____; corn seed designated *rmr9-1* and having ATCC Accession No. _____; corn seed designated *rmr7-2* and having ATCC Accession No.

_____; corn seed designated *rmr11-1* and having ATCC Accession No. _____; corn seed designated *Mop1-4* and having ATCC Accession No. _____ corn seed designated *cc2343* and having ATCC Accession No. _____and corn seed designated *Mop1-5* and having ATCC Accession No. _____.

[0026] The present invention is also directed to a corn plant having all of the phenotypic and morphological characteristics of a plant produced from corn seed selected from the seed designated *Mop1-1* and having ATCC Accession No. ____; corn seed designated *Mop1-2EMS* and having ATCC Accession No. _____; corn seed designated *Mop2-1* and having ATCC Accession No. _____ ; corn seed designated *mop3-1* and having ATCC Accession No. _____ ; corn seed designated *rmr1-1* and having ATCC Accession No. _____; corn seed designated *rmr1-2* and having ATCC Accession No. _____; corn seed designated *rmr2-1* and having ATCC Accession No. _____; corn seed designated *rmr7-1* and having ATCC Accession No. _____; corn seed designated *rmr6-1* and having ATCC Accession No. _____; corn seed designated *rmr8-1* and having ATCC Accession No. _____; corn seed designated *rmr9-1* and having ATCC Accession No. _____; corn seed designated *rmr7-2* and having ATCC Accession No. _____; corn seed designated *rmr11-1* and having ATCC Accession No. _____; corn seed designated *Mop1-4* and having ATCC Accession No. _____ ;corn seed designated *cc2343* and having ATCC Accession No. _____ and corn seed designated *Mop1-5* and having ATCC Accession No. _____.

[0027] The present invention is directed to an ovule or pollen of a plant having all of the phenotypic and morphological characteristics of a plant produced from corn seed where the corn seed is selected from the corn seed designated *Mop1-1* and having ATCC Accession No. ____; corn seed designated *Mop1-2EMS* and having ATCC Accession No. ____; corn seed designated *Mop2-1* and having ATCC Accession No. _____ ; corn seed designated *mop3-1* and having ATCC Accession No. _____ ; corn seed designated *rmr1-1* and having ATCC Accession No. _____; corn seed designated *rmr1-2* and having ATCC Accession No. _____; corn seed designated *rmr2-1* and having ATCC Accession No. _____; corn seed designated *rmr7-1* and having ATCC Accession No. _____; corn seed designated *rmr6-1* and having ATCC Accession No. _____; corn seed designated *rmr8-1* and having ATCC Accession No. _____; corn seed designated *rmr9-1* and having ATCC Accession No. _____; corn seed designated *rmr7-2*and having ATCC Accession No. _____; corn seed designated *rmr11-1* and having ATCC Accession No. _____; corn seed designated *Mop1-4* and having ATCC Accession No. _____ corn seed designated *cc2343* and having ATCC Accession No. _____and corn seed designated *Mop1-5* and having ATCC Accession No. _____.

[0028] The present invention is further directed to progeny seed produced from crossing a plant grown from a seed selected from the group of corn seed including corn seed designated *Mop1-1* and having ATCC Accession No. _____; corn seed designated *Mop1-2EMS* and having ATCC Accession No. _____; corn seed designated *Mop2-1* and having ATCC Accession No. _____ ; corn seed designated *mop3-1* and having ATCC Accession No. _____ ; corn seed designated *rmr1-1* and having ATCC Accession No. _____; corn seed designated *rmr1-2* and having ATCC Accession No. _____; corn seed designated *rmr2-1* and having ATCC Accession No. _____; corn seed designated *rmr7-1* and having ATCC Accession No. _____; corn seed designated *rmr6-1* and having ATCC Accession No. _____; corn seed designated *rmr8-1* and having ATCC Accession No. _____; corn seed designated *rmr9-1* and having ATCC Accession No. _____; corn seed designated *rmr7-2* and having ATCC Accession No. _____; corn seed designated *rmr11-1* and having ATCC Accession No. _____; corn seed designated *Mop1-4* corn seed designated *cc2343* and having ATCC Accession No. _____ and having ATCC Accession No. _____ and corn seed designated *Mop1-5* and having ATCC Accession No. _____ ; with another corn plant and the corn seed produced therefrom.

[0029] The present invention is further directed to tissue culture of regenerable cells of corn plants selected from *Mop1-1*; *Mop1-2EMS*; *Mop2-1*; *mop3-1*; *rmr1-1*; *rmr1-2*; *rmr2-1*; *rmr7-1*; *rmr6-1*; *rmr8-1*; *rmr9-1*;*rmr7-2*; *rmr11-1*; *Mop1-4*, *cc2343* and *Mop1-5* and seeds derived therefrom, wherein the tissue culture regenerates plants capable of expressing all the physiological and morphological characteristics of the corn plants selected from *Mop1-1*; *Mop1-2EMS*; *Mop2-1*; *rmr1-1*; *rmr1-2*; *rmr2-1*; *rmr7-1*; *rmr7-2*; *rmr6-1*; *rmr8-1rmr9-1*;*cc2343* ; *rmr11-1*; *Mop1-4*; *Mop1-5* and seeds derived therefrom, respectively.

[0030] The tissue culture of the invention may include regenerable cells comprising cells derived from embryos, immature embryos, meristematic cells, immature tassels, microspores, pollen, leaves, anthers, roots, root tips, silk, flowers, kernels, ears, cobs, husks, stalks, protoplasts or callus.

[0031] The present invention is further directed to corn plants regenerated from the tissue culture of regenerable cells of the invention.

[0032] The present invention is further directed to a process of producing corn seed, comprising crossing a first parent corn plant with a second parent corn plant, wherein the first or second corn plant is a corn plant selected from *Mop1-1*, *Mop1-2EMS*; mop-2-1; mop-3-1; *rmr1-1*; *rmr1-2*; *rmr2-1*; *rmr7-1*; *rmr7-2*; *rmr6-1*; *rmr8-1*; *rmr9-1*; *rmr11-1*; *cc2343*; *Mop1-4* and *Mop1-5*. The process of producing the corn seed of the invention comprises the steps of: (a)

planting in pollinating proximity seeds of the first and second corn plants; (b) cultivating the seeds of the first and second corn plants into plants that bear flowers; (c) emasculating the male flowers of the first or second corn plant to produce an emasculated corn plant; (d) allowing cross-pollination to occur between the first and second corn plants; and (e) harvesting seeds produced on the emasculated corn plant.

[0033] The process of producing the corn seed of the invention also includes hand-pollinations that can be carried out on a large scale. Briefly, female styles are protected from stray pollen by placing small bags over the developing ear shoots, pollen is collected from tassels and placed on the silks of selected plants.

[0034] The present invention is further directed to a method of producing hybrid corn plants and hybrid corn seed using the process of the invention and F1 hybrid seed produced therefrom.

BRIEF DESCRIPTION OF THE DRAWINGS

[0035] The invention will be better understood by reference to drawings in which:

[0036] Figure 1 shows phenotypes and crosses demonstrating paramutation. Figure 1A shows seed phenotypes of *r1* haplotypes. The top panels are the phenotypes of the parents, the paramutable, *R-r* haplotype or the paramutagenic *R-st* and *R-mb* haplotype. It does not matter which parent is used as male versus female. The central panel shows the phenotype of the F1 seeds. When these are planted and crossed as male to colorless recessive null *r1* alleles (not shown), the resulting seed phenotypes segregate (third panel). *R-st* and *R-mb* segregate unchanged. *R-r* is changed to a lower expressing form, *R-r'*. Typically *R-st* is more paramutagenic than *R-mb*, as *R-r'* is less pigmented when segregating from a *R-st* versus a *R-mb* F1. Figure 1B shows plant and anther phenotypes of *b1* and *pl1* alleles, respectively. Plant phenotypes of the parents are shown in the top panels, and the F1 in the left middle panels, directly below the parents. The progeny resulting from crosses of F1 plants back to paramutable alleles are shown in the bottom panels. The only genotype that segregates is *B'* or *Pl'*. The asterisk is used to indicate the newly paramutagenic allele, which is fully capable of altering a paramutable allele.

[0037] Figure 2 shows structures of genes that undergo paramutation. Coding regions are indicated by black horizontal arrows. The coding and promoter regions are not to scale. Different promoter regions are indicated by distinct boxes. Promoter regions with sequence similarity are indicated by the same boxes: solid, striped or open. Figure 2A shows the structure of the *r1* genes within paramutable, *R-r:standard* and *R-d:Catspaw*, and

paramutagenic haplotypes, *R*-stippled and R-*marbled*. The *doppia* sequences are indicated by the open boxes. The distance between the *r1* genes is not indicated. The approximate size and location of the transposable elements in *R-st* and *R-mb* are indicated by triangles. Figure 2B shows the structure of the *b1* and *pl1* alleles that undergo paramutation. The 3' end of the transcribed region of *pl1* is part of a repeat containing the 3' flanking region. The location of the *doppia* related sequences in *Pl'* and *Pl-Rh* is indicated by an open box.

[0038] Figures 3A-3F show the phenotypes associated with the *Mop1-1* mutation. The genotypes of the photographed plants are indicated below each panel. (A) *B' Mop1/Mop1-1*, (B) *B' Mop1-1/Mop1-1*, (C) *B-I Mop1/Mop1*, (D) *B' Mop1-1/Mop1-1* plant with *B'*-like sectors, (E) *Pl' Mop1/-* (either *Mop1/Mop1* or *Mop1/Mop1-1*), (F) *Pl' Mop1-1/Mop1-1*.

[0039] Figures 4A and 4B show diagrams outlining tests for heritability of *B'* and *Pl'* from homozygous *Mop1-1* individuals.(A) If *Mop1-1* heritably alters *B'* to *B-I*, then *B' Mop1-1/Mop1-1* individuals would generate *B-I Mop1-1* gametes and progeny would be dark plants. Alternatively, if *B'* is still *B'*, then all gametes would be *B' Mop1-1*, *B'* would paramutate *B-I* [indicated by (*B-I*)'] in the next generation and all progeny would be light. (B) If *Mop1-1* heritably alters *Pl'* to *Pl-Rh*, then *Pl' Mop1-1/Mop1-1* individuals would generate *Pl-Rh Mop1-1* gametes and progeny would have dark anthers. Alternatively, if *Pl'* is still *Pl'*, then all gametes would be *Pl' Mop1-1*, *Pl'* would paramutate *Pl-Rh* [indicated by (*Pl-Rh*)'] in the next generation and all progeny would have light anthers.

[0040] Figure 5 shows anther color scores (ACSs) of *Pl' Mop1-1/Mop1-1* outcrosses vs. *Pl' Mop1/Mop1-1* outcrosses. *Pl' Mop1-1/Mop1-1* individuals versus *Pl' Mop1/Mop1-1* individuals were outcrossed to *Pl-Rh* (*Mop1/Mop1*) testers. Individual progeny plants were scored for amount of anther pigment.

[0041] Figure 6 shows the amounts of transcripts in *Mop1-1* versus Wild-Type Siblings. An example of RNase protections for *b1*, *pl1* and actin on four sibling individuals is shown. All individuals are homozygous *B'* and *Pl'*, and segregating for *Mop1-1* as indicated. The bar graph shows the normalized amounts of *b1* (open bars) and *pl1* (closed bars) RNA levels from the RNase protection.

[0042] Figures 7A-7C show transcription rates in *mop1-1/mop1-1* versus *Mop1-1/mop1-1* siblings. (A) An example of an in vitro transcription assay showing the SK+ plasmid negative control and signal for *b1*, c2, and ubiquitin2 transcription in *B' Mop1-1/Mop1-1* versus *B' Mop1/Mop1-1* individuals. (B) Pair-wise data for *b1* transcription rate from several in vitro transcription assays for several *Mop1-1/Mop1-1* (closed bars) versus *Mop1/Mop1-1*

(open bars) individuals. The data represent three separate comparisons between pairs of sibling individuals. The n-fold (designated x) increase is given below for each pairwise comparison. (C) Pair-wise data from RNase protection assays for *b1* RNA levels normalized to ubiquitin2 RNA levels for the same *Mop1-1/Mop1-1* (closed bars) versus *Mop1/Mop1-1* (open bars) sibling individuals as examined in (B).

[0043] Figure 8 shows a diagram showing three progeny classes from *B'*/B-Peru *Mop1/Mop1-1* self-pollinations. *B' Mop1-1* plants were crossed with B-Peru *Mop1* plants. The light F1 *B'*/B-Peru *Mop1/Mop1-1* plants were self-pollinated, and purple kernels (B-Peru/-) were planted. The progeny fell into three phenotypic classes; the numbers and genotypes of each phenotype are shown. The *Mop1* genotype of the 24 B-Peru/B-Peru plants was determined by a testcross with *B' Mop1-1*.

[0044] Figure 9 shows a diagram outlining a test for the ability of *Mop1-1* to prevent *b1* paramutation. *B'/B' Mop1-1/Mop1-1* plants were crossed to *B-I*/b *Mop1/Mop1-1* plants, generating four types of segregating progeny. The *B'/B-I* and *B'*/b progeny were distinguished by restriction fragment length polymorphisms. The *B'*/b progeny were not analyzed further. Both classes of *B'/B-I* progeny (dark and light) were crossed with testers heterozygous for *B-I*/B-Peru, *Mop1/Mop1* (and with testers null for *b1*, not diagrammed) to test whether the *B-I* allele heterozygous with *B'* had become *B'* in the *Mop1-1/Mop1-1* versus *Mop1/Mop1-1* plants. The expectations for crosses with *B'/B-I Mop1-1/Mop1-1* with one type of tester (B-Peru *Mop1* which gives purple kernels) are shown. The expectation for the *B'/B-I Mop1/Mop1-1* progeny is that all offspring will be light plants (not diagrammed).

[0045] Figures 10A-10B show a summary of *Mop1-1* effects on *r1* paramutation. (A) Parental genotypes and progeny classes used to evaluate the effect of *Mop1-1* on the establishment of *r1* paramutation. *Mop1* genotypes were determined based upon the intensity of plant pigment within the Pl/- phenotypic class. Individuals belonging to the two *R-d/-* genotypic classes indicated were identified by crossing to an *R-g* tester stock and the *R-d/R-g* kernel progeny were then assayed for pigment intensity. (B) The bar graph shows results of paramutation test with *r1*. Color scores for kernels inheriting the *R-d* allele are plotted from individuals having the parental genotype indicated along the x-axis. The first two columns are *Mop1/-* genotype controls. Color score equals 100 minus average reflectometer reading as described (Alleman and Kermicle, 1993). Error bars indicate SD.

[0046] Figures 11A-11D show phenotypes characteristic of *Mop1* mutations relative to Wild-Type siblings. (A) A *B' Mop1/Mop1-1* individual bearing a normal tassel. (B) A *B' Mop1-1/Mop1-1* individual bearing a feminized tassel (strong tasselseed). (C) A *B'/- Mop1-*

2EMS/Mop1-2EMS individual bearing a severely barrenized tassel. (D) A runty *B' Mop1-1/Mop1-1* individual in which the feminized terminal inflorescence failed to emerge.

[0047] Figures 12A-12B show DNA blots assaying methylation of repeated sequences. Individual genotypes are indicated above the DNA blots (*B'* stands for *B'/B' Mop1/Mop1*). (A) Samples digested with the methylation insensitive enzyme BstNI (B) and the methylation sensitive enzyme EcoRII (E) were probed with the 45S ribosomal repeat. (B) Samples digested with HpaII (H) or MspI (M) were probed with the centromere repeat.

[0048] Figure 13 shows the methylation status of *MuDR* and *Mu1* elements in a family segregating for *MuK* and *Mop1-1*. A) A HinfI digest of DNA from this family probed with a *Mu1* internal fragment. The lanes marked "*Mop1*" (lanes 1-4) indicate samples from plants that were homozygous for *Mop1-1*. The lanes marked "wild type" (lanes 5-21) indicate samples from plants that were either *Mop1-1/Mop1* or *Mop1/Mop1*. The hypomethylated *Mu1* and slightly larger *Mu1*.7 kb fragments are indicated. B) To control for complete digestion by the restriction enzyme, the blot shown in A was stripped and rehybridized with a fragment of the a1 gene. C) A SacI digest of the same DNA samples as are shown in panel A, probed with an internal *MuDR* fragment. The 4.8 kb *MuDR* internal fragment diagnostic for the presence of hypomethylated, intact *MuDR* elements is indicated. The individual in lane 20 was retested and the presence of methylated *Mu1* elements was confirmed.

[0049] Figure 14 shows the reversal of methylation of *Mu1* elements in a family segregating for *Mop1-1*. A) A blot of a HinfI digest of DNA from a family segregating *Mop1-1* homozygotes ("*Mop1*", lanes 1-5) and *Mop1-1/Mop1-1* heterozygotes ("wild type", lanes 6-19) was probed with a *Mu1* fragment. B) To control for complete digestion by the restriction enzyme, the blot shown in A was stripped and rehybridized with a fragment of the a1 gene. C) The same blot probed with the BglII/SalI fragment from a region upstream of the *b1* gene (see Figure 4d). D) Gel blot analysis of DNA from progeny from the self-fertilization of the individual in lane 6 of panel A, a *Mop1-1/Mop1* individual with methylated *Mu1* elements. DNA from this family was digested with HinfI and probed with *Mu1*. Lanes marked with asterisks contain DNA from *Mop1-1* homozygous individuals, as determined by pigment phenotype.

[0050] Figure 15 shows the hypomethylation of *Mu1* in the absence of *MuDR*. A) A HinfI digest of DNA from a family segregating for *Mop1-1/Mop1-1* ("*Mop1-1*", lanes 1-3) and *Mop1-1/Mop1* or *Mop1/Mop1* ("wild type", lanes 4-6). The resulting blot was hybridized with the *Mu1* internal probe. B) DNA from the same family digested with SacI and probed with an internal fragment of *MuDR*. The diagnostic fragment for a full length *MuDR* element is as

indicated. Digestions with methylation insensitive enzymes confirmed no intact *MuDR* elements were present (data not shown). In both panels, the last lane contains DNA from a *MuDR*-active individual.

[0051] Figure 16 shows gel blots of digests of DNA from mutant ("*Mop1*") and wild type ("w.t.") individuals using several methyl-sensitive enzymes. A) ApaI/BamHI. Lanes 1-7 are *Mop1-1* homozygotes. Lanes 8-14 are heterozygous siblings. Lanes 15 and 16 are *Mop1-1* homozygous and wild type individuals digested with only BamHI. B) SalI/BamHI. Lanes 1-4 are *Mop1-1* homozygotes, lanes 5-8 are wild type. C) PvuII/BamHI. Lanes 1-4 are *Mop1-1* homozygotes, lanes 5-8 are wild type. D) Restriction map of the region upstream of the start of transcription of *B'* and *B-I* (the sequence of these alleles are identical in this region). Sites marked with an asterisk are partially or completely methylated in plants carrying *B' Mop1/Mop1*; *B-I Mop1/Mop1*, or *Mop1/Mop1-1*; or *B' Mop1-1/Mop1-1*. The other sites are unmethylated in all genotypes tested. A = ApaI, B = BamHI, Pv = PvuII, H = HinfI, Sa = SalI. The bar above the map indicates the probe.

[0052] Figure 17 shows an outline of a genetic screen used to identify mutations affecting seedling pigmentation. *Pl-Rh* seedlings have fully colored first leaf sheaths (top right). *Pl'* seedlings have very weakly colored first leaf sheaths (top left). Pollen from *Pl-Rh* plants was treated with ethyl methanesulfonate (EMS) and brushed on the silks of *Pl'* plants. All *Pl'/Pl-Rh* M1 plants had a *Pl'* seedling phenotype (middle panel). M1 plants were self pollinated and M2 families were screened for *Pl-Rh*-like seedlings. The bottom four panels represent a M2 family segregating 3:1 for *Pl'* and *Pl-Rh*-like seedlings.

[0053] Figures 18A-18C show how EMS-derived mutations affect anther pigment and *pl1* RNA levels. (A) Anther phenotypes of plants that are heterozygous (top three variegated anthers) and homozygous (bottom three fully colored anthers) for the ems235 (*rmr1-2*) allele. The difference between anther pigment phenotypes was identical for both the ems96 (*rmr2-1*) and ems136 (*rmr1-1*) alleles (not shown). (B) RNase-protection assay measuring the levels of *pl1* and actin RNA found in anther tissues of plants with the indicated genotypes. (C) Histogram of RNase-protection results for *pl1* RNA levels measured relative to actin RNA for the indicated genotypes. Bars represent average measurements for each genotype and crossbars above each bar indicate the standard error. The number of samples measured for each genotype is as follows: ems96/+, n = 2; ems96/ems96, n = 2; ems136/+, n = 3; ems136/ems136, n = 4; ems235/+, n = 5; ems235/ems235, n = 6.

[0054] Figures 19A-C show that *Pl'* can change to *Pl-Rh* in plants that are homozygous for rmr mutations. (A) Pedigree outlining two genetic crosses used to show that plants

homozygous for the rmr mutations can transmit non-paramutagenic *Pl-Rh* alleles. Pl(') is used to identify a *pl1* allele that should have been *Pl'* in the first generation but is not paramutagenic; the Pl(')/*Pl-Rh* plants in the second cross have fully colored anthers. (B) Results of crosses initiated with plants that were homozygous for the *rmr1-1* allele. Histogram represents the number of plants with a given anther color score that were either sm1/sm1 (salmon colored silks; closed bars) or Sm1/sm1 (yellow silks; open bars). (C) Results of crosses initiated with plants that were homozygous for the *rmr2-1* allele.

[0055] Figure 20 shows anther phenotypes of plants that are homozygous for the pl-A632 allele. Photographs are of tassels from an A632 plant (left), a plant that is homozygous for the *rmr1-1* allele (center), and a plant that is homozygous for the *rmr2-1* allele (right).

[0056] Figure 21 A-B show results of in vitro transcription reactions comparing *B-I/B-I*; *Pl'/Pl'*; *rmr1-1/rmr1-1* and *B-I/B-I*; *Pl'/Pl'*; *rmr1-1/rmr1* (closed bars) and *B-I/B-I*; *Pl'/Pl'*; *rmr1-1/rmr1-1* and *B-I/B-I*; *Pl'/Pl'*; *rmr1-1/Rmr1* (open bars) husk tissues. (A) shows one example of the primary results while (B) represents combined results from 5 independent experiments. Experimental design and representation of results are as described in Hollick et. al 2000.

[0057] Figure 22 A-B show results of in vitro transcription reactions comparing *B-I/B-I*; *Pl'/Pl'*; *rmr2-1/rmr2-1* (closed bars) and *B-I/B-I*; *Pl'/Pl'*; *rmr2-1/Rmr2* (indicated as *rmr2-1/+*) (open bars) husk tissues. (A) is one example of the primary results while (B) represents combined results from 5 independent experiments. Experimental design and representation of results are as described in Hollick et. al 2000.

[0058] Figure 23 shows a diagram indicating the identification of a dominant mutation. The fact that paramutation always occurs when *B-I* and *B'* are heterozygous, and that this phenotype is apparent in all F1 individuals means that one can efficiently screen for dominant mutations that disrupt the establishment of paramutation. An exceptional dark plant was identified among numerous light siblings. This individual was found to carry a dominant mutation, referred to as *Mop2-1*. This original dark plant was outcrossed to *gl2 B'* wt, and progeny of this cross were crossed to *gl2 B-I* wt. Following the marked chromosomes in these crosses demonstrate that this mutation is loosely linked to *b1*, mapping to the short arm of chromsome 2.

[0059] Figure 24 shows the segregation of *B-I* phenotypes among progeny of *gl2 B-I* wt x *Mop2-1 B'*. *B'* individuals heterozygous for *Mop2-1* were crossed to *B-I* and progeny were scored for sheath and tassel pigment levels. A color score of 1-3 is typical of a *B'* individual, and 6-7 is typical of a *B-I* individual. The distribution of color scores among progeny from

nine *B'* individuals heterozygous for the *Mop2-1* mutation crossed to *B-I* are shown graphically. Some families show a clear bimodal distribution whereas others have a higher frequency of intermediate individuals.

[0060] Figure 25 shows the phenotypes associated with homozygous *Mop2-1* individuals and heterozygous siblings. A) Heterozygous *Mop2-1* individual showing normal *B'* pigmentation. B) Homozygous *Mop2-1* individual showing the intensification of pigment and tassel seed phenotype.

[0061] Figure 26 shows an RNA blot demonstrating differences in *b1* transcript levels between mutants and their wild-type siblings. A) Comparison of *b1* RNA levels in *Mop2-1/Mop2-1* versus *Mop2-1/+* husk tissue. B) Comparison of *b1* RNA levels in *mop3-1/mop3-1* versus *mop3-1/+* husk tissue. *mop1-1/mop1-1* versus *mop1-1/+* husk samples were also included on this blot as a control. C) Comparison of *b1* RNA levels in husk tissue from *CC2343* lights versus darks husk tissue. All plants were *B'*. RNA isolation was as described in Example 1 and preparation of the RNA blot, probe and hybridization conditions were as described in Patterson et al. 1993.

[0062] Figures 27A-F show phenotypes associated with the *mop3-1* (A,B) and *CC2343* (C-F) mutants. Figures 27A shows the darkly pigmented phenotype of *mop3-1/mop3-1 B'*. Figure 27B shows the phenotypes associated with Mop3/*mop3-1*, which has the same phenotype as plants homozygous for the wild type allele. Figure 27C shows dark and light plants in a *CC2343* family. Note how much taller the light plants are relative to the dark plants. Figure 27D shows a medium dark plant segregating in a *CC2343* family. Figure E and F show a dark plant without a tassel and a medium dark plant with a partially feminized tassel from cc2343 families.. These phenotypes are also seen in *mop3-1* families.

[0063] Figure 28 shows the constructs used to generate the transgenic lines.

[0064] Figures 29A-D show the phenotypes associated with *Mop1-1*, *Mop2-1*, *rmr1-1* or *rmr2-1* activation of a previously transcriptionally silent 35S *B-I* genomic transgene. Figure 29E shows *rmr2-1* activation of a previously silent BBBS transgene. The phenotype was the same with *Mop1-1* and *rmr1-1* and the BBBS transgene. The genotypes of each plant are indicated below each panel.

[0065] Figure 30 shows that transgene activation by the *Mop1-1*, *rmr1-1* and *rmr2-1* mutations is associated with increased transgene transcript levels. The top panel is an RNA blot examining transgene transcipt levels in individuals heterozygous for the mutation versus individuals homozygous for each of the mutations. The blot was probed with a region of the

b1 gene. The bottom panel is a picture of the ethidium stained gel that was blotted, showing that similar levels of total RNA were added to each lane. See Patterson et al, 1993 for methods.

[0066] Figure 31 summarizes data from in vitro transcription assays demonstrating that transgene activation in *Mop1-1* and *rmr2-1* mutants is at the transcriptional level. Methods are as described in Dorweiler et al. 2000.

[0067] Figure 32 A-B show methylation gel blot analyses of *Mu1* sequences for (A) rm*r1*, rmr6, and (B) rmr2 mutants.

[0068] Figure 33 A-B show comparison of (A) *rmr6-1/Rmr6* (indicated as *rmr6-1/+)* and (B) *rmr6-1/rmr6-1* mature tassel phenotypes.

[0069] Figure 34A-C show molecular genetic expression analyses of *rmr6-1/rmr6-1* and *rmr6-1/Rmr6* (indicated as *rmr6-1/+)* materials. (A) RNase protection results carried out as described in Example 3. B-C show results of in vitro transcription reactions comparing *B-I/B'*; *Pl'/Pl'*; *rmr6-1/rmr6-1* (closed bars) and *B-I/B-I*; *Pl'/Pl'*; *rmr6-1/+* (open bars) husk tissues. (B) is one example of the primary results while (C) represents combined results from 5 independent experiments.

DETAILED DESCRIPTION OF THE INVENTION

[0070] In order to more fully understand the invention, the following definitions are provided:

[0071] **AFLP marker:** An Amplified Restriction Fragment Length Polymorphism is a marker used in genetic mapping.

[0072] **Allele:** Any of one or more alternative forms of a gene locus, all of which alleles relate to one trait or characteristic. In a diploid cell or organism, the two alleles of a given gene occupy corresponding loci on a pair of homologous chromosomes.

[0073] **Anther Color Score (ACS):** A graded quantitative score between 1 and 7 describing relative amounts of anthocyanin pigment found in anthers of maize plants expressing *Pl'* or *Pl-Rh* alleles. ACS 1 describes anthers with virtually no pigment, ACS 7 describes fully-pigmented anthers, and ACS 2-6 describe intermediate levels of variegated pigment.

[0074] **Backcrossing:** A process in which a breeder repeatedly crosses hybrid progeny back to one of the parents, for example, a first generation hybrid (F1) with one of the parental genotypes of the F1 hybrid.

[0075] **Crossing:** The pollination of a female flower of a corn plant, thereby resulting in the production of seed from the flower.

[0076] **Cross-pollination:** Fertilization by the union of two gametes from different plants.

[0077] **Diploid:** A cell or organism having two sets of chromosomes.

[0078] **Emasculate:** The removal of plant male sex organs or the inactivation of the organs with a chemical agent or a cytoplasmic or nuclear genetic factor conferring male sterility.

[0079] **Expression:** The expression of a gene refers to the production of the RNA or protein encoded by the gene. For protein encoding genes, this involves transcription of the gene into RNA and translation of the RNA into protein. Gene expression can be monitored by examining RNA levels, examining transcription rates, or by measuring protein levels.

[0080] **Epigenetics:** Epigenetics refers to altered gene expression associated with alternative chromatin and/or methylation states superimposed upon an unchanged primary DNA sequence.

[0081] **F1 Hybrid:** The first generation progeny of the cross of two plants.

[0082] **Gene Silencing:** Repression of gene activity via inhibited transcription and/or by increased RNA degradation.

[0083] **Genetic Complement:** An aggregate of nucleotide sequences, the expression of which sequences defines the phenotype in corn plants, or components of plants including cells or tissue.

[0084] **Genotype:** The genetic constitution of a cell or organism.

[0085] **Haploid:** A cell or organism having one set of the two sets of chromosomes in a diploid.

[0086] **Heterosis:** A synonym for "hybrid vigor" where the superiority of the offspring of a cross between two stocks to the better of the parents. Also used as a synonym for "heterozygote advantage" which is a relation between alleles in which the heterozygote, Aa, is superior to either homozygote, AA or aa. Heterosis in the latter sense is often invoked to explain hybrid vigor.

[0087] **Linkage:** A phenomenon wherein alleles on the same chromosome tend to segregate together more often than expected by chance if their transmission was independent.

[0088] **Maize:** Maize (Zea mays L.) is often referred to as corn in the United States. Maize, can be bred by both self-pollination and cross-pollination techniques. Maize has separate male and female flowers on the same plant, located on the tassel and the ear, respectively. Natural pollination occurs in maize when the wind blows pollen from the tassels to the silks that protrude from the tops of ears. Maize and corn are used interchangeably throughout this specification.

[0089] **Maize Nomenclature:** Maize nomenclature is the means to identify maize genes and alleles. A gene is designated with lower case italics (*b1*) in maize nomenclature [See also Trends in Genetics "Genetic Nomenclature Guide" (1995) or the web site http://www.agron.missouri.edu/maize nomenclature.html]. Specific alleles are indicated with an allele designation separated from the gene designation with a hyphen. Dominant alleles are indicated by an upper case gene designation (B-Peru) and recessive alleles by a lower case gene designation (*b1*-K55). Gene products are indicated by all caps and are not italicized (B).

[0090] **Marker:** A readily detectable phenotype or molecular or biochemical characteristic, preferably inherited in codominant fashion (both alleles at a locus in a diploid heterozygote are readily detectable), preferably with no environmental variance component, i.e., heritability of 1. Examples of markers include AFLPs, SNPs and SSLP markers.

[0091] **Methylation:** Process by which methyl groups are added to certain nucleotides in genomic DNA. Methyl sensitive restriction enzymes, such as *Sac*I and *Hin*fI cannot cut sequences with certain specific methylations. Methylation of DNA is often associated with reduced gene activity.

[0092] **Mutant Corn Plant:** A plant that is homozygous for a recessive or dominant mutation in a particular gene or heterozygous for a dominant mutation in a particular gene.

This mutation is usually associated with a change in the DNA sequence of the gene that is altered.

[0093] **Paramutation:** A mitotically and meiotically heritable change in the transcriptional activity of a gene that can occur spontaneously or be induced by a paramutagenic allele. Paramutation is the change in gene activity of one allele due to its exposure to another allele. For example, at the *b1* locus in maize, strong activity of the *B-I* allele confers dark purple pigmentation throughout the plant while weak activity of the *B'* allele confers light pigment. In a *B-I/B'* heterozygote, *B-I* is invariably changed to *B'*; only*B'* alleles segregate from the heterozygote (Figure 1). The sensitive allele (*B-I*) is described as paramutable and the inducing allele (*B'*) is described as paramutagenic. Similarly, with paramutation at *pl1*, *Pl-Rh* is paramutable and *Pl'* is paramutagenic (see Figure 1).

[0094] **Establishment of Paramutation:** The ability of a paramutagenic allele to change a paramutable allele into a paramutagenic allele. For example, when *B'* is crossed with *B-I*, *B-I* is always changed to *B'*, paramutation is always established when these two alleles are combined in a heterozygote.

[0095] **Maintenance of Paramutation:** The ability of a paramutant state (such as *B'*) to be maintained. For example, when plants containing *B'* or *Pl'* are grown, the low transcription level and the ability to be paramutagenic is maintained in those plants.

[0096] **Heritable Maintenance of the Paramutant State:** The ability of a paramutant state (such as *B'* or *Pl'*) to be maintained in subsequent generations. When *B'* or *Pl'* are sexually transmitted, the low transcription level and the ability to be paramutagenic is maintained in progeny plants.

[0097] **Reduction in the establishment of Paramutation:** In wild type genetic backgrounds when *B'* is crossed with *B-I*, when *Pl'* is crossed with *Pl-Rh*, or when *R-st* is crossed with *R-d*, paramutation always occurs, it is always established. For the *b1* and *r1* loci over 100,000 different events at these two loci have been examined in the 50 years this has been studied. For *pl1*, several thousand events have been examined in the 10 years this has been studied. In the presence of certain mutations, paramutation is prevented. For example, in every plant that was homozygous for *Mop1-1* (Example 1), the establishment of paramutation was completedly prevented; it was reduced by 100%.

[0098] **Reduction in the maintenance of Paramutation:** When *B'* is in wild type genetic backgrounds, paramutation is always maintained: *B'* always shows reduced transcription and strong paramutagenic activity. In the presence of certain mutations, such

as *mop1-1*, the transcription rate and RNA levels of *B'* are dramatically increased (Example 1).

[0099] **Reduction in the heritable maintenance of the Paramutant State:** When *Pl'* is sexually transmitted from *Pl'* homozygotes in wild type genetic backgrounds, paramutation is always maintained: *Pl'* shows reduced transcription and strong paramutagenic activity. In certain mutant plants, the RNA levels from *Pl'* are increased, and in addition, nonparamutagenic *pl1* alleles are sexually transmitted from homozygous *Pl'* genotypes.

[00100] **Paramutagenic alleles:** Paramutagenic alleles induce a change in sensitive, paramutable alleles. Examples include *B'*, *Pl'* and *R-st*.

[00101] **Paramutable alleles:** Paramutable alleles are alleles that are sensitive to paramutagenic alleles. Examples include *B-I*, *Pl-Rh*, and *R-d*. Following paramutation, sensitive alleles are termed paramutant (or paramutated) and designated with an apostrophe (i.e., generically, *B'*; *Pl'*; R', etc.) In the examples of *b1* and *pl1* paramutation, once the paramutable alleles becomed paramutated they are paramutagenic.

[00102] **Neutral alleles:** Neutral alleles neither induce nor respond to paramutation.

[00103] **Progeny Plant:** A plant produced from a parental line by crossing or selfing.

[00104] **Phenotype:** The detectable characteristics of a cell or organism, which characteristics are the manifestation of the genetic makeup of the organism or cell and the environment.

[00105] **Regeneration:** The development of a plant from tissue culture.

[00106] **RFLP genetic marker profile:** A profile of band patterns of DNA fragment lengths typically separated by agarose gel electrophoresis after restriction endonuclease digestion of DNA.

[00107] **Self-pollination:** The transfer of pollen from the anther to the stigma of the same plant.

[00108] **Single Locus Converted (Conversion) Plant:** Plants which are developed by a plant breeding technique called backcrossing wherein essentially all of the desired morphological and physiological characteristics of an inbred are recovered in addition to the

characteristics conferred by the single locus transferred into the inbred via the backcrossing technique. A single locus may comprise one gene, or in the case of transgenic plants, one or more transgenes integrated into the host genome at a single site (locus).

[00109] **SSLP marker:** A Short Sequence Length Polymorphism marker is a marker utilized in mapping.

[00110] **SNP marker:** A Single Nucleotide Polymorphism is a marker utilized in mapping.

[00111] **Tissue Culture:** A composition comprising isolated cells of the same or a different type or a collection of such cells organized into parts of a plant.

[00112] **Transgene:** A genetic sequence that has been introduced into the nuclear, or chloroplast genome of a maize plant by a genetic transformation technique to produce a transgenic plant.

[00113] **Transgene Silencing:** Repression of transgene activity via inhibited transcription and/or increased RNA degradation.

[00114] Taking into account these definitions, the present invention relates to the isolation and characterization of corn mutants altered in the establishment or maintenance of paramutation, an example of gene silencing.

Screening System

[00115] Genetic and molecular studies of the *Zea mays* L. corn (maize) *b1* (booster 1) and *pl1* (purple plant 1) loci are utilized in this invention to isolate new corn lines with reduced gene silencing activity. The *b1* gene encodes a transcriptional regulator required for anthocyanin pigment synthesis. Two particular *b1* alleles show strong allelic interactions that lead to heritable changes in *b1* gene activity. Paramutated and non-paramutated phenotypes can be distinguished on the basis of seedling pigment (2-3 weeks old), immature plants and mature plants. The *pl1* gene encodes a different transcriptional regulator also required for anthocyanin pigment synthesis. Two particular *pl1* alleles show a strong allelic interaction that leads to heritable changes in *pl1* gene activity. Paramutated and non-paramutated phenotypes can be distinguished on the basis of seedling pigment (14-18 days post-imbibition), mature plants and anthers of mature plants. The visual nature of the assay at an early growth stage and throughout development, and the non-essential nature of the anthocyanin pathway make it an excellent system for studying gene silencing. Most of the genes encoding both the specific biosynthetic enzymes and the transcriptional regulators

have been cloned and sequenced. Pigment levels are a simple and sensitive indicator of quantitative changes in RNA accumulation of these transcriptional regulatory loci. The *b1* and *r1* loci encode functionally duplicate basic-helix-loop-helix (bHLH) transcription factors and the *pl1* and c1 loci encode functionally duplicate Myb-like transcription factors. Production of pigment co-requires the function of both a bHLH (*b1* or *r1*) and a Myb (*pl1* or c1) factor. Since alleles of all four loci have distinctive tissue-specific expression patterns, it is the specific combination of various alleles that determine whether or not pigment is produced in any given tissue.

Paramutation

[00116] All examples of paramutation involve an interaction between alleles that leads to a heritable reduction in the expression of one of the alleles. Alleles sensitive to altered expression are termed paramutable, and alleles inducing the change, paramutagenic. Following paramutation, sensitive alleles are termed paramutant (or paramutated) and designated with an apostrophe (i.e., generically, *B'*, *Pl'*, R' etc.). Many alleles at *r1*, *b1* and *pl1* do not participate in paramutation; these alleles have been referred to in the literature as either neutral or non-paramutagenic. Table 1 contains a summary of the loci, the paramutagenic and paramutable alleles and the tissues where paramutation is typically monitored at each locus.

TABLE 1. Summary of Loci Discussed

Locus	Paramutable Form	Paramutagenic Form	(Strength)	Tissue Where Paramutation Monitored
rl	*R-r:std*	*R-r:std*[a]	(weak)	aleurone of seeds.
rl	*R-d*	*R-d*[a]	(weak)	aleurone, coleoptile and roots of seedlings
rl		*R-st, R-sc*[b]	(strong)	aleurone of seeds
rl		*R-mb, R-scm*[c]	(strong)	aleurone of seeds
bl	*B-I*	*B'*	(strong)	epidermal cells in most vegetative tissues, auricle in seedlings, pericarp, cob
pll	*Pl-Rh*	*Pl'*	(strong)	anthers, first leaf sheath of young seedling, epidermal cells in most vegetative tissues

[00117] [a]The paramutable alleles, *R-r:std* and *R-d* become weakly paramutagenic after being heterozygous with strongly paramutagenic alleles.

[00118] [b]*R-st* contains the four *r1* genes indicated in Figure 2A. The *I-R* element in the *Sc* gene causes a stippled phenotype in the aleurone (Figure 1A). *R-sc* is equivalent to *R-st*, except that the *I-R* element has been lost from the *Sc* gene restoring full color to the aleurone. There is no difference in paramutagenic strength between *R-st* and *R-sc* derivatives that maintain four *r1* genes.

[00119] [c]*R-mb* contains the three *r1* genes indicated in Figure 2A. The *Shooter* element in the *Scm* gene causes a marbled phenotype in the aleurone (Figure 1A). *R-scm* is equivalent to *R-mb,* except that the *shooter* element has been lost from the *Scm* gene restoring full color to the aleurone. There is no difference in paramutagenic strength between *R-mb* and *R-scm* derivatives that maintain three *r1* genes.

Paramutation Phenotypes

[00120] Paramutation was first studied with the paramutable *R-r*:standard (*R-r:std*) haplotype, which pigments seed and plant parts. In *R-r:std*, seed pigmentation is considerably more sensitive to paramutation than plant pigmentation (Brink and Mikula 1958), while in another paramutable haplotype that pigments both seed and plant parts, *R-d*:Catspaw (*R-d*), both seed and plant pigmentation levels are sensitive to paramutation (Brink et al., 1970). Haplotype is used to describe the particular *r1* "alleles", because all are complex containing multiple genes, resulting in the classic definition of allele being inaccurate. In the most extensively studied *r1* haplotypes, neither plant nor seed expression is markedly reduced in F1 plants (Figure 1A). Consequently, silencing of paramutable *r1* haplotypes typically is monitored by crossing the F1 plants with null, recessive testers and analyzing kernel pigment levels in the progeny. The phenotype is strongest if the F1 is used as male. The fact that many, but not all, paramutable *r1* haplotypes also undergo genomic imprinting (reflected in weaker pigment expressed in the endosperm if transmitted through the male) increases the sensitivity of this assay. Paramutagenic *r1* haplotypes, *R-st*ippled (*R-st*) and R-marbled (*R-mb*), are expressed in seeds, although both alleles also are expressed in the scutellum and coleoptile tip. Seed phenotypes of parental, F1 and testcross progeny are shown in Figure 1A for *R-st*, *R-mb* and *R-r:std*. At *b1* and *pl1* reduced expression is always seen in the F1 and the reduced expression is always transmitted to progeny (Coe, 1966; Hollick et al., 1995). The F1 plants typically have the phenotype of the paramutagenic *B'* or *Pl'* alleles. Figure 1B shows the phenotypes of parental, F1 and progeny plants undergoing paramutation at *b1* and *pl1*.

[00121] Following meiosis, paramutant alleles retain the reduced expression state as discussed above and are themselves paramutagenic; they induce reduced expression of

naive (not previously exposed to paramutagenic alleles) paramutable alleles in subsequent heterozygotes. At *r1*, a paramutable haplotype is changed into a weakly paramutagenic haplotype after it segregates from the paramutagenic haplotype. In the *r1* literature, this is termed secondary paramutation to distinguish it from the stronger paramutagenicity seen with *R-st* and *R-mb*. After one generation of heterozygosity with *R-st*, the paramutagenicity of *R-r:std* is considerably weaker than the paramutagenicity of *R-st* (Brown and Brink, 1960). In contrast, at the *b1* and *pl1* loci, newly altered paramutagenic alleles (*B'* or *Pl'*) are strongly paramutagenic. They induce heritable silencing (paramutagenicity) of paramutable alleles indistinguishably from the parental paramutagenic alleles (Coe, 1966; Hollick et al., 1995). This led Coe to describe the phenomenon at *b1* as a conversion event (Coe, 1959).

[00122] Two assays are routinely used to monitor paramutation: 1) the ability of a paramutagenic allele to cause a heritable reduction in the expression of a paramutable allele; and 2) the heritable alteration of the paramutant allele into a paramutagenic allele. At *b1* and *pl1* these two phenotypes always occur simultaneously and completely. In contrast, at *r1* the extent of paramutagenicity obtained by a paramutant haplotype depends on the circumstances of the crosses, as discussed in detail in Chandler, et al., (2000).

Structures of paramutable and paramutagenic alleles

[00123] Paramutagenic and paramutable *r1* haplotypes share *r1* coding sequences and parts of the promoter regions, but these coding and promoter regions are arranged in structurally distinct ways (Figure 2A). The paramutagenic alleles at *r1* are readily distinguishable from the paramutable alleles; by these structural differences and by distinct expression patterns (Figure 1A). The paramutable alleles *R-r:std* and *R-d* each contain inverted duplications of *r1* coding regions (*S1/S2* and *D1/D2*) flanking a region called Sigma. In *R-r:std*, *Sigma* is the seed-specific promoter for the *S1* (seed1) and S2 (seed2) genes in *R-r:std* (Walker et al., 1995). *Sigma* in *R-r:std* contains rearranged pieces of sequences that have structural features common to transposable elements (these have been named *doppia*, and are indicated by open boxes in Figure 2A) together with a small amount of sequence homologous to the *P* (plant) promoter (indicated in Figure 2A as hatched areas; Walker et al., 1995). In *R-d*, Sigma contains only *doppia*-related sequences (R. Okagaki and J. Kermicle, Genbank U93178). The Sigma regions are located at the same sites relative to *S2* and *D2* in *R-r:std* and *R-d*, respectively (Figure 2A), but some sequences of *D1* are missing relative to *S1* (Walker et al., 1995; R. Okagaki and J. Kermicle, Genbank U93178), suggesting that the alleles may have arisen by independent events from a common progenitor. Additionally, *R-r:std* contains an *r1* promoter region denoted *q* adjacent to a second *doppia*-related sequence between *S1* and *P*. Ten kbp of sequence separates *q* from *S1* and 190 kbp of sequence

separate *q* and *P* (Robbins et al, 1991, Walker et al., 1995). In *R-r:std*, *S1/S2* are expressed only in the seed, *P* in vegetative parts of the plant and q not at all since it is lacking an *r1* coding region (Walker et al., 1995). The *q* sequences are structurally very similar to the *P* promoter region (Walker et al., 1995), and when linked to an *r1* coding region by unequal crossing over, activate expression in the same plant parts as P (Dooner, 1979; J. Kermicle, M. Alleman and W. Eggleston, unpublished data). In *R-d*, *D1/D2* are expressed in both plant and seed (Bray and Brink, 1966; R. Okagaki and J. Kermicle, personal communication).

[00124] Both well-studied paramutagenic haplotypes contain multiple *r1* genes (Figure 2A) with distinct patterns of expression. The paramutagenic haplotype *R-st* contains four *r1* genes in direct orientation (Eggleston et al., 1995). Three distinct *r1* genes were cloned from the *R-mb* haplotype (Panavas et al., 1999). Quantitative analysis of the products of unequal recombination of *R-mb* demonstrates that this haplotype contains the three cloned *r1* genes organized in direct repeats (Neal, 1998; M. Alleman, personal communication) as inferred by Panavas et al., (1999).

[00125] The Sc (self color) and Scm (self color marbled) genes in *R-st* and *R-mb*, respectively (Figure 1A), pigment the aleurone, scutellum and tip of the coleoptile in dried seeds, although Scm has much higher scutellurn and coleoptile pigment than does Sc (A. Eggleston, M. Alleman, J. Kermicle and W. Eggleston, unpublished data). Scm but not Sc also pigments the scutellar node of germinating seeds (A. Eggleston, M. Alleman and W. Eggleston, unpublished data). The I-R transposable element within Sc, and the Shooter element within Scm, cause the stippled and marbled phenotypes, respectively (Eggleston et al., 1995; Panavas et al., 1999). Full purple color is observed when the elements are lost by excision, gene conversion or unequal crossing over. The full color haplotypes are referred to as R-sc and R-scm. Neither I-R nor Shooter significantly contributes to paramutagenicity; losses of I-R from *R-st* and Shooter from *R-mb* by excision or gene conversion events that maintain the number of *r1* genes have no effect on paramutagenicity (Kermicle et al., 1995; Quinn, 1999; J. Kermicle and W. Eggleston, unpublished data).

[00126] In addition to the highly expressed Sc and Scm genes, both *R-st* and *R-mb* contain additional *r1* genes with distinct expression patterns. The Nc (near colorless) genes in *R-st* (Figure 2A) are weakly expressed in the aleurone of seeds (Eggleston et al., 1995). The Lcm (Lc-like marbled) genes in *R-mb* do not contain functional promoters as they are not expressed in any tissue unless linked to a different, active promoter. Intriguingly, each Nc gene in *R-st* contains a *doppia* sequence in the promoter-proximal region in the same relative orientation and position as the *doppia* sequences in Sigma adjacent to S2 and D2 in *R-r:std* and *R-d* (W. Eggleston, unpublished data; Matzke et al., 1996). However, the *doppia*

sequences are unlikely to be necessary for paramutagenicity as the Lcm genes in *R-mb* do not contain *doppia* or Sigma sequences in their promoter proximal regions (Panavas et al., 1999; J. Kermicle and W. Eggleston, unpublished data).

[00127] In contrast to *r1*, where paramutable and strongly paramutagenic haplotypes are structurally distinct, the *b1* and *pl1* paramutagenic alleles arose through spontaneous changes of paramutable to paramutagenic alleles. Extensive restriction map analyses comparing the paramutable and paramutagenic forms of these alleles have identified no distinguishing differences (DNA insertions, deletions or other rearrangements) between the two types. The regions examined include ~25 kbp spanning the 4 kbp *b1* coding region (Patterson et al., 1993) and ~10 kbp spanning the 1 kbp *pl1* coding region. The structures of these alleles are shown in Figure 2B. In *pl1* there is a region of 290 bp that shares sequence similarity with *doppia*, located upstream of the transcription initiation site. However, this sequence is not sufficient for *pl1* paramutation as it is found in non-paramutagenic alleles as well (reviewed in Hollick et al., 1997). No *doppia* sequences have been detected in any *b1* allele (V. Chandler, unpublished data).

Paramutation can occur spontaneously

[00128] Paramutable alleles of all three loci, *B-I*, *Pl-Rh*, and *R-r:std*, are inherently unstable, as they change to lower expression states even in the absence of paramutagenic alleles. However, whether the reduced expression correlates with the simultaneous acquisition of paramutagenicity differs significantly among the loci. At *b1*, spontaneous changes of *B-I* to *B'* occur at high frequencies (often 1-10%), and are readily detected as lightly colored sectors within a *B-I* plant or lightly pigmented *B'* progeny arising from homozygous *B-I* plants (Coe, 1966). Spontaneously derived *B'* alleles are as fully paramutagenic as *B'* alleles segregating from *B'/B-I* plants. Thus, the lower expression state associated with *B'* is invariably associated with strong paramutagenicity that results in paramutation when crossed with paramutable alleles (Coe, 1966). At *pl1* spontaneous changes of *Pl-Rh* to *Pl'* also occur at high frequencies, but there is variation in the expression levels and paramutagenic strength of the spontaneous derivatives (Hollick et al., 1995). Alleles with the most reduction in expression are strongly paramutagenic, while alleles with intermediate expression are weakly paramutagenic (Hollick et al., 1995; Hollick et al., 2000). Spontaneous changes to lower gene expression states do occur with paramutable *r1* alleles, but these states are not heritable nor are they associated with acquisition of strong paramutagenicity (Brink et al., 1968).

Expression levels are influenced by allele interactions

[00129] The stability of the expression states differs for the *b1*, *pl1* and *r1* loci. The paramutable *B-I* allele is extremely unstable when homozygous, changing into *B'* at very high frequencies, whereas when *B-I* is heterozygous with alleles that do not participate in paramutation it is much more stable (Coe, 1966; V. Chandler and K. Kubo, unpublished data). The paramutant, now paramutagenic allele, *B'*, is very stable as it has not been found to change back to a highly expressed, paramutable form in standard maize stocks (Coe, 1966; Patterson et al., 1995), independent of whether it is carried homozygous or heterozygous with other alleles. In contrast to the stability of *B'*, paramutant *Pl'* and R' are metastable. Their expression states fall within a wide continuum of levels in subsequent generations. The frequency and direction of changes depend on whether they are maintained as homozygotes or heterozygotes and the nature of the other allele, as reviewed in Chandler et al. 2000.

Paramutation occurs in somatic cells

[00130] The light color phenotype and low levels of *b1* or *pl1* RNA in F1 individuals containing paramutagenic and paramutable alleles, combined with the spontaneous change from *B-I* to *B'* or *Pl-Rh* to Pl' in somatic cells, suggest *b1* and *pl1* paramutation occurs in somatic cells (reviewed in Chandler et al., 1996). Several lines of evidence also suggest that paramutation at *r1* occurs somatically. Sastry et al. (1965) showed that individual tassel branches of a single plant transmitted distinct expression states, demonstrating somatic sectors. Treatment of F1 seeds with irradiation (Shih and Brink, 1969) or alkylating agents (Axtell and Brink, 1967; Brink et al., 1968) results in significant reductions in paramutation. Further, *r1* paramutation is significantly impacted by altering the environmental conditions of young F1 seedlings at times well before meiosis (Mikula, 1995).

Genetic Strategies to Identify Trans-acting Components Required for Paramutation.

[00131] To understand the underlying mechanisms that control paramutation, an example of gene silencing, it will be essential to identify the molecular machinery responsible for the establishment and maintanence of paramutation. This invention is directed to the use of several mutational approaches to identify trans acting components required for paramutation.

[00132] Mutations were isolated from several distinct genetic screens (described below) designed to identify mutations that cause increase pigment production in *B'/B'* and /or *Pl'/Pl'* plants. Mutations identified in screens using the *B'/B'* system are designated mop mutations for mediator of paramutation and those identified in screens using *Pl'/Pl'* materials are designated rmr mutations for required to maintain repression. There are at least two putative

functions for the mop and rmr gene products; 1) they could be required to establish paramutation, 2) they could be required to maintain gene repression previously established by paramutation. The genes could also be required for both establishment and maintenance of paramutation. In addition, rmr and mop gene products could either be required to maintain repressed gene expression only during somatic development or they could be required to ensure that the repressed expression state is also transferred to progeny (maintained through meiosis, gametophyte development, fertilization and embryo development). Distinctive seedling and plant pigment phenotypes facilitate genetic screens for mutations that alter either the establishment or the maintenance of paramutation. Screens have been developed using either the *b1* (Example 1) or the *pl1* system (Example 3). Both transposon (Example 1) and EMS mutagenesis (Example 3, 6) have been utilized. The extreme penetrance of *b1* and *pl1* paramutation (it always occurs) together with the stability of the paramutant state at both loci make these powerful screens. The basic approach is to mutagenize the parent containing the paramutagenic allele, cross to the paramutable allele, and then screen the resulting M1 generation for rare dominant mutants that prevent the establishment of paramutation. These are identified as rare dark seedlings or plants among lightly pigmented siblings. To screen for recessive mutations that fail to maintain the reduced expression state associated with paramutation, M1 plants are self-pollinated and the resulting M2 are screened for darkly pigmented seedlings or plants. The background for the *b1* screen is extremely low, as the only two dark exceptions in the approximately 10,000 M1 *B'/B-I* plants screened were subsequently shown to be dominant or semi-dominant mutants. The background with the *pl1* screen is higher, as the examination of approximately 50,000 M1 individuals, revealed 0.2% dark seedlings that when grown to maturity had light *Pl'* anthers, so were not true mutants. To date 16 independent mutations have been identified that relieve the gene silencing associated with the paramutant state (described in detail in Examples below).

Phenotypes of Corn Mutants with Reduced Gene Silencing Activity

[00133] Several methods have been used to characterize the various mutant alleles isolated from these genetic screens. 1) Genetic complementation tests establish potential allelism (determines the number of unique genes identified) and reveal potential genetic interactions between different genes. 2) RNA levels of *B'*, *Pl'* or both, are determined in the mutants and compared to wild type siblings. 3) The relative impact each mutation has on maintaining the silenced expression state of *B'*, *Pl'* or both, is determined by monitoring the stability of the reduced expression state in progeny from outcrosses. 4) Genetic experiments are done to assess the ability of the mutations to prevent the establishment of paramutation at *b1*, *r1* and *pl1*. 5) Phenotypic analysis compares the relative impact each mutation has on

normal maize plant growth and development. 6) The ability of the mutants to affect other epigenetic phenomena such as transposon methylation and transgene silencing is tested.

Plant Breeding

[00134] The goal of field crop breeding is to combine various desirable traits in a single variety/hybrid. The reduced gene silencing traits of the mutant corn lines of the present invention can be introduced into corn breeding programs in order to prevent the establishment or maintenance of gene silencing in new corn lines.

[00135] Breeding techniques take advantage of a plant's method of pollination. There are two general methods of pollination: a plant self-pollinates if pollen from one flower is transferred to the same or another flower of the same plant. A plant cross-pollinates if pollen comes to it from a flower on a different plant.

[00136] Corn plants (Zea mays L.) can be bred by both self-pollination and cross-pollination. Both types of pollination involve the corn plant's flowers. Corn has separate male and female flowers on the same plant, located on the tassel and the ear, respectively. Natural pollination occurs in corn when wind blows pollen from the tassels to the silks that protrude from the tops of the ear shoot.

[00137] Plants that have been self-pollinated and selected for type over many generations become homozygous at almost all gene loci and produce a uniform population of true breeding progeny, a homozygous plant. Here, the mutant plants of the invention have been selfed and selected for the absence of gene silencing.

[00138] A cross between two homozygous plants produces a uniform population of hybrid plants that are heterozygous for many gene loci. Conversely, a cross of two plants each heterozygous at a number of loci produces a population of hybrid plants that differ genetically and are not uniform. The resulting non-uniformity makes performance unpredictable.

[00139] The development of uniform corn plant hybrids requires the development of homozygous inbred plants, the crossing of these inbred plants, and the evaluation of the crosses. Pedigree breeding and recurrent selection are examples of breeding methods used to develop inbred plants from breeding populations. Those breeding methods combine the genetic backgrounds from two or more inbred plants such as the mutant plants of this invention or various other broad-based sources into breeding pools from which new inbred plants are developed by selfing and selection of desired phenotypes. The new inbreds are

crossed with other inbred plants and the hybrids from these crosses are evaluated to determine which of those have commercial potential.

Pedigree Breeding

[00140] The pedigree breeding method involves crossing two genotypes. In this invention, one genotype would be a mutant corn plant of the invention. Each genotype can have one or more desirable characteristics lacking in the other; or, each genotype can complement the other. If the two original parental genotypes do not provide all of the desired characteristics, other genotypes can be included in the breeding population. Superior plants that are the products of these crosses are selfed and selected in successive generations. Each succeeding generation becomes more homogeneous as a result of self-pollination and selection. Typically, this method of breeding involves five or more generations of selfing and selection.

[00141] Backcrossing can also be used to improve an inbred plant. Backcrossing transfers a specific desirable trait from one inbred or non-inbred source to an inbred that lacks that trait. In this invention, the reduced gene silencing activity trait of the corn lines of the invention can be transferred to another corn line. This can be accomplished, for example, by first crossing a superior inbred (A) (recurrent parent) to a donor inbred (non-recurrent parent), which carries the appropriate locus or loci for the trait in question. In the present invention, the mutants of the invention would serve as the donor stock either in inbred or non-inbred genetic backgrounds. The progeny of this cross are then mated back to the superior recurrent parent (A) followed by selection in the resultant progeny for the desired trait to be transferred from the non-recurrent parent. After five or more backcross generations with selection for the desired trait, the progeny are heterozygous for loci controlling the characteristic being transferred, but are like the superior parent for most or almost all other loci. The last backcross generation would be selfed to give pure breeding progeny for the trait being transferred.

[00142] A single cross hybrid corn variety is the cross of two inbred plants, each of which has a genotype that complements the genotype of the other. The hybrid progeny of the first generation is designated F1. Typically, F1 hybrids are more vigorous than their inbred parents. This hybrid vigor, or heterosis, is manifested in many polygenic traits, including markedly improved yields, better stalks, better roots, better uniformity and better insect and disease resistance. In the development of hybrids only the F1 hybrid plants are typically sought. An F1 single cross hybrid is produced when two inbred plants are crossed. A double

cross hybrid is produced from four inbred plants crossed in pairs (A x B and C x D) and then the two F1 hybrids are crossed again (A x B) X (C x D).

Development of F1 Lines

[00143] The development of a hybrid corn variety generally involves three steps: (1) the selection of plants from various germplasm pools such as the mutant corn plants of the invention; (2) the selfing of the selected plants for several generations to produce a series of inbred plants, which, although different from each other, each breed true and are highly uniform; and (3) crossing the selected inbred plants with unrelated inbred plants to produce the hybrid progeny (F1). During the inbreeding process in corn, the vigor of the plants decreases. Vigor is restored when two unrelated inbred plants are crossed to produce the hybrid progeny (F1). An important consequence of the homozygosity and homogeneity of the inbred plants is that the hybrid between any two inbreds is always the same. Once the inbreds that give a superior hybrid have been identified, hybrid seed can be reproduced indefinitely as long as the homogeneity of the inbred parents is maintained. Conversely, much of the hybrid vigor exhibited by F1 hybrids is lost in the next generation (F2). Consequently, seed from hybrid varieties is not used for planting stock. It is not generally beneficial for farmers to save seed from F1 hybrids. Rather, farmers purchase F1 hybrid seed for planting every year.

[00144] In selecting a second plant to cross with the mutant corn plants of the invention for the purpose of developing novel inbred lines, it will typically be desired to choose those plants which either themselves exhibit one or more selected desirable characteristics or which exhibit the desired characteristic(s) when in hybrid combination. Examples of potentially desired characteristics include greater yield, better stalks, better roots, resistance to insecticides, herbicides, pests, and disease, tolerance to heat and drought, reduced time to crop maturity, better agronomic quality, higher nutritional value, and uniformity in germination times, stand establishment, growth rate, maturity, and fruit size. Alternatively, the mutant corn plants of the invention may be crossed with a second, different inbred plant for the purpose of producing hybrid seed that is sold to farmers for planting in commercial production fields. In this case, a second inbred variety is selected which confers desirable characteristics when in hybrid combination with the first inbred line.

[00145] In a preferred embodiment, crossing comprises the steps of: (a) planting in pollinating proximity seeds of a first and a second parent corn plant, and preferably, seeds of a first inbred corn plant and a second, distinct inbred corn plant; (b) cultivating or growing the seeds of the first and second parent corn plants into plants that bear flowers; (c)

emasculating flowers of either the first or second parent corn plant, i.e., treating the flowers so as to prevent pollen production, or alternatively, using as the female parent a male sterile plant, thereby providing an emasculated parent corn plant; (d) allowing natural cross-pollination to occur between the first and second parent corn plants; (e) harvesting seeds produced on the emasculated parent corn plant; and, (f) growing the harvested seed into a corn plant, preferably, a hybrid corn plant.

[00146] Parental plants are typically planted in pollinating proximity to each other by planting the parental plants in alternating rows, in blocks or in any other convenient planting pattern. Where the parental plants differ in timing of sexual maturity, it may be desired to plant the slower maturing plant first, thereby ensuring the availability of pollen from the male parent during the time at which silks on the female parent are receptive to pollen. Plants of both parental parents are cultivated and allowed to grow until the time of flowering. Advantageously, during this growth stage, plants are in general treated with fertilizer and/or other agricultural chemicals as considered appropriate by the grower.

[00147] At the time of flowering, in the event that the mutant plants of the invention are employed as the male parent, the tassels of the other parental plant are removed from all plants employed as the female parental plant to avoid self-pollination. The detasseling can be achieved manually but also can be done by machine, if desired. Alternatively, when the female parent corn plant comprises a cytoplasmic or nuclear gene conferring male sterility, detasseling may not be required. Additionally, a chemical gametocide may be used to sterilize the male flowers of the female plant. In this case, the parent plants used as the male may either not be treated with the chemical agent or may comprise a genetic factor which causes resistance to the emasculating effects of the chemical agent. Gametocides affect processes or cells involved in the development, maturation or release of pollen. Plants treated with such gametocides are rendered male sterile, but typically remain female fertile. The use of chemical gametocides is described, for example, in U.S. Pat. No. 4,936,904, the disclosure of which is specifically incorporated herein by reference in its entirety. Furthermore, the use of ROUNDUP□ herbicide in combination with glyphosate tolerant maize plants to produce male sterile corn plants is disclosed in PCT Publication WO 98/44140.

[00148] Following emasculation, the plants are then typically allowed to continue to grow and natural cross-pollination occurs as a result of the action of wind, which is normal in the pollination of grasses, including corn. As a result of the emasculation of the female parent plant, only the pollen from the male parent plant is available for pollination because tassels, and thereby pollen bearing flowering parts, have been previously removed from all plants of the inbred plant being used as the female in the hybridization. Of course, during this

hybridization procedure, the parental varieties are grown such that they are isolated from other corn fields to minimize or prevent any accidental contamination of pollen from foreign sources. These isolation techniques are well within the skill of those skilled in this art.

[00149] Both parental inbred plants of corn may be allowed to continue to grow until maturity or the male rows may be destroyed after flowering is complete. Only the ears from the female inbred parental plants are harvested to obtain seeds of a novel F1 hybrid. The novel F1 hybrid seed produced can then be planted in a subsequent growing season in commercial fields or, alternatively, advanced in breeding protocols for purposes of developing novel inbred lines.

[00150] In the present invention, the mutant corn lines of the present invention can be used as inbred lines. It is more likely, however, that the mutant lines of this invention will be used in breeding programs to introduce the gene silencing repression traits into other lines. The gene silencing repression traits may be introduced into other lines by the use of single locus conversion.

Single Locus Conversions

[00151] When the term inbred corn plant is used in the context of the present invention, this also includes any single locus conversions of that inbred. The term single locus converted plant as used herein refers to those corn plants that are developed by a plant breeding technique called backcrossing wherein essentially all of the desired morphological and physiological characteristics of an inbred are recovered in addition to the single locus transferred into the inbred via the backcrossing technique. Backcrossing methods can be used with the present invention to improve or introduce a characteristic such as reduced gene silencing activity into the inbred. The term backcrossing as used herein refers to the repeated crossing of a hybrid progeny back to one of the parental corn plants for that inbred. The parental corn plant that contributes the locus or loci for the desired characteristic is termed the nonrecurrent or donor parent. This terminology refers to the fact that the nonrecurrent parent is used one time in the backcross protocol and therefore does not recur. The parental corn plant to which the locus or loci from the nonrecurrent parent are transferred is known as the recurrent parent as it is used for several rounds in the backcrossing protocol. In a typical backcross protocol, the original inbred of interest (recurrent parent) is crossed to a second nonrecurrent parent stock (inbred or otherwise) that carries the single locus of interest to be transferred. The resulting progeny from this cross are then crossed again to the recurrent parent and the process is repeated until a corn plant is obtained wherein essentially all of the desired morphological and physiological characteristics of the recurrent

parent are recovered in the converted plant, in addition to the single transferred locus from the nonrecurrent parent. The backcross process may be accelerated by the use of molecular markers, such as RFLP, SSLP, SNP or AFLP markers to identify plants with the greatest genetic complement from the recurrent parent, but retaining the single locus of interest.

[00152] The goal of a backcross protocol is to alter or substitute a single trait or characteristic such as repression of gene silencing or reduced gene silencing activity in the original inbred. To accomplish this, a single locus of the recurrent inbred is modified or substituted with the desired locus from the nonrecurrent parent, while retaining essentially all of the rest of the desired genetic, and therefore the desired physiological and morphological constitution of the original inbred. Here, the mutants with reduced gene silencing can be used as the nonrecurrent parent. The exact backcrossing protocol will depend on the characteristic or trait being altered to determine an appropriate testing protocol. Although backcrossing methods are simplified when the characteristic being transferred is a dominant allele, a recessive allele may also be transferred. In this instance it may be necessary to introduce a test of the progeny to determine if the desired characteristic has been successfully transferred. It may also be possible to follow transfer of the allele with reduced gene silencing properties using tightly linked molecular markers, such as SSLP or RFLP.

Tissue Cultures and in Vitro Regeneration of Corn Plants

[00153] A further aspect of the invention relates to tissue cultures of the mutant corn plants of the invention. As used herein, the term "tissue culture" indicates a composition comprising isolated cells of the same or a different type or a collection of such cells organized into parts of a plant. Exemplary types of tissue cultures are protoplasts, calli and plant cells that are intact in plants or parts of plants, such as embryos, pollen, flowers, kernels, ears, cobs, leaves, husks, stalks, roots, root tips, anthers, silk, and the like. In a preferred embodiment, the tissue culture comprises embryos, protoplasts, meristematic cells, pollen, leaves or anthers derived from immature tissues of these plant parts. Means for preparing and maintaining plant tissue cultures are well known in the art (U.S. Pat. No. 5,538,880; and U.S. Pat. No. 5,550,318, each incorporated herein by reference in their entirety). By way of example, a tissue culture comprising organs such as tassels or anthers has been used to produce regenerated plants (U.S. Pat. No. 5,445,961 and U.S. Pat. No. 5,322,789; the disclosures of which are incorporated herein by reference).

Tassel/Anther Culture

[00154] Tassels contain anthers that in turn enclose microspores. Microspores develop into pollen. For anther/microspore culture, if tassels are the plant composition, they are

preferably selected at a stage when the microspores are uninucleate, that is, include only one, rather than 2 or 4 nuclei. Methods to determine the correct stage are well known to those skilled in the art and include mitramycin fluorescent staining, trypan blue and acetocarmine squashing. The mid-uninucleate microspore stage has been found to be the developmental stage most responsive to the subsequent methods disclosed to ultimately produce plants.

Lines For Crossing

[00155] The mutant corn lines of the invention may be crossed with any other suitable corn plants. Such suitable corn plants generally include commercially useful traits.

Deposit Information

[00156] Representative of, but not limiting the invention, Applicants have deposited the following seeds with the American Type Culture Collection.

[00157] Applicants have made available to the public without restriction a deposit of at least 2500 seeds of corn *Mop1-1* with the American Type Culture Collection (ATCC), Rockville, MD 20852, which has been assigned ATCC number XX and deposited on _____..

[00158] Applicants have made available to the public without restriction a deposit of at least 2500 seeds of corn *Mop1-2EMS* with the American Type Culture Collection (ATCC), Rockville, MD 20852, which has been assigned ATCC number XX and deposited on _____.

[00159] Applicants have made available to the public without restriction a deposit of at least 2500 seeds of corn *Mop2-1* with the American Type Culture Collection (ATCC), Rockville, MD 20852, which has been assigned ATCC number XX and deposited on _____.

[00160] Applicants have made available to the public without restriction a deposit of at least 2500 seeds of corn *mop3-1* with the American Type Culture Collection (ATCC), Rockville, MD 20852, which has been assigned ATCC number XX.

[00161] Applicants have made available to the public without restriction a deposit of at least 2500 seeds of corn *CC2343* with the American Type Culture Collection (ATCC), Rockville, MD 20852, which has been assigned ATCC number XX.

[00162] Applicants have made available to the public without restriction a deposit of at least 2500 seeds of corn *rmr1-1* with the American Type Culture Collection (ATCC), Rockville, MD 20852, which has been assigned ATCC number XX and deposited on _____.

[00163] Applicants have made available to the public without restriction a deposit of at least 2500 seeds of corn *rmr1-2* with the American Type Culture Collection (ATCC), Rockville, MD 20852, which has been assigned ATCC number XX and deposited on _____.

[00164] Applicants have made available to the public without restriction a deposit of at least 2500 seeds of corn *rmr2-1* with the American Type Culture Collection (ATCC), Rockville, MD 20852, which has been assigned ATCC number XX and deposited on _____.

[00165] Applicants have made available to the public without restriction a deposit of at least 2500 seeds of corn *rmr7-1* with the American Type Culture Collection (ATCC), Rockville, MD 20852, which has been assigned ATCC number XX and deposited on _____.

[00166] Applicants have made available to the public without restriction a deposit of at least 2500 seeds of corn *rmr6-1* with the American Type Culture Collection (ATCC), Rockville, MD 20852, which has been assigned ATCC number XX and deposited on _____.

[00167] Applicants have made available to the public without restriction a deposit of at least 2500 seeds of corn *rmr8-1* with the American Type Culture Collection (ATCC), Rockville, MD 20852, which has been assigned ATCC number XX and deposited on _____.

[00168] Applicants have made available to the public without restriction a deposit of at least 2500 seeds of corn *rmr9-1* with the American Type Culture Collection (ATCC), Rockville, MD 20852, which has been assigned ATCC number XX and deposited on _____.

[00169] Applicants have made available to the public without restriction a deposit of at least 2500 seeds of corn *Mop1-4* with the American Type Culture Collection (ATCC), Rockville, MD 20852, which has been assigned ATCC number XX and deposited on _____.

[00170] Applicants have made available to the public without restriction a deposit of at least 2500 seeds of corn *Mop1-5* with the American Type Culture Collection (ATCC), Rockville, MD 20852, which has been assigned ATCC number XX and deposited on _____.

[00171] Applicants have made available to the public without restriction a deposit of at least 2500 seeds of corn *rmr7-2* with the American Type Culture Collection (ATCC), Rockville, MD 20852, which has been assigned ATCC number XX and deposited on _____.

[00172] Applicants have made available to the public without restriction a deposit of at least 2500 seeds of corn *rmr11-1* with the American Type Culture Collection (ATCC), Rockville, MD 20852, which has been assigned ATCC number XX and deposited on _____.

[00173] The deposits will be maintained in the ATCC depository, which is a public depository, for a period of 30 years, or 5 years after the most recent request, or for the effective life of the patent, whichever is longer, and will be replaced if a deposit becomes nonviable during that period.

[00174] Although the foregoing invention has been described in some detail by way of illustration and examples for purposes of clarity and understanding, it will be obvious that certain modifications and alternative embodiments of the invention are contemplated which do not depart from the spirit and scope of the invention as defined by the foregoing teachings and appended claims.

EXAMPLES

Example 1

Isolation of *Mop1-1* Mutant Plant.

[00175] **General.** Paramutation is an interaction between two specific alleles, which leads to a heritable alteration in one of the alleles at a very high frequency. Paramutation was first described at the *r1* locus of maize (Brink, 1956; Brink, 1958). Brink observed that aleurone pigment levels in genotypically identical kernels (triploid endosperm *R-r/R-g/R-g*) were significantly different, dependent upon whether *R-r* was previously homozygous or heterozygous with *R-stippled* (*R-st*). *R-st* (termed paramutagenic) induced a heritable reduction in the expression of standard *R-r* (termed paramutable); altered *R-r* is designated

R-r' (reviewed in Kermicle, 1996). The paramutable *b1* and *pl1* alleles are *B-I* and *Pl-Rh* respectively, and when heterozygous with a paramutagenic *B'* or *Pl'* allele, the paramutable allele is heritably changed into a paramutagenic allele (Coe, 1966; Hollick et al., 1995). The paramutable *B-I* and *Pl-Rh* alleles are also unstable, spontaneously changing to *B'* and *Pl'* (Coe, 1966; Hollick et al., 1995). Most alleles of *b1*, *r1* and *pl1* do not participate in paramutation and are termed neutral. Paramutation-like phenomena are not restricted to pigment regulatory genes; they have also been described in other plants (reviewed in Brink, 1973), and with transgenes in Petunia and tobacco (Meyer et al., 1993; Matzke et al., 1994; reviewed in Hollick et al., 1997).

[00176] Paramutation has been extensively characterized at three maize loci: *r1*, *b1*, and *pl1* (For review see Chandler et al., 2000). *b1* and *r1* encode functionally interchangeable basic-helix-loop-helix (bHLH) factors (Styles et al., 1973; Ludwig et al., 1989; Goff et al., 1990; Radicella et al., 1991), whereas *pl1* encodes a myb-related transcription factor (Cone et al., 1993). Activation of the anthocyanin biosynthesis pathway requires co-expression of a bHLH factor and a myb-related factor (Goff et al., 1992). In general, the *pl1* locus is expressed in the plant, whereas its functional equivalent, c1, is expressed in the embryo and the aleurone layer of the kernel endosperm. Therefore, when functional alleles of *pl1* and c1 are present, it is generally the *b1* and *r1* alleles present that determine the tissue-specific patterns of anthocyanin expression (Styles et al., 1973).

[00177] **Plant Stocks.** All plant stocks contained dominant functional alleles for all the genes encoding the anthocyanin biosynthetic enzymes required in vegetative plant tissues. Because transcription of these genes in vegetative plant tissues is controlled by *pl1* in combination with *b1* or *r1*, the specific *b1*, *pl1* and *r1* alleles are indicated for relevant stocks. One exception is the distinction between *Pl'-mahogany* (*Pl'*) and *Pl-Rhoades* (*Pl-Rh*) (Hollick et al., 1995). Many stocks possess the *R-g* allele of *r1* (no expression in the seed or plant), which precludes reliable scoring of *Pl'* versus *Pl-Rh*. In these stocks, we have used *Pl* to indicate the presence of either *Pl-Rh*, or its spontaneous derivative *Pl'*.

[00178] Though stocks containing various *b1* alleles have been maintained in the laboratory of Vicki Chandler (now at the University of Arizona) for several years, they were originally obtained from a variety of sources: *B-I PIR-g* (inbred W23 background), *B' PIR-g* (inbred K55 background), and *b1*-K55 *PIR-g* (inbred K55 background) stocks from E.H. Coe, Jr. (University of Missouri, Columbia), B-bar from E.D. Styles (University of Victoria), and B-Peru (inbred W22 background) from G. Neuffer (University of Missouri). Jay Hollick and Vicki Chandler have maintained *Pl-Rh* and *Pl'-mahogany* stocks originally obtained from E.H. Coe, Jr. and additional *Pl-Rh* stocks from the Maize Cooperation Stock Center. J.L. Kermicle has

maintained the *R-stippled* (*R-st*), *R-r*:standard (*R-r:std*, a specific accession of *R-r*, also known as standard *R-r*), and *R-d*:*Catspaw* (*R-d*) stocks (each containing *B-bar* and *pl-W22*, inbred *W22* background) established by R.A. Brink and colleagues.

[00179] **Genetic Screen.** A *B-I PIR-g* stock was used to generate a *B-I PIR-g* Mu stock by sequential backcrosses into active *Mutator* stocks (Patterson et al., 1991). The *B-I PIR-g* Mu stock, carrying functional alleles for all of the anthocyanin biosynthetic enzymes, was crossed to *B' PIR-g* (inbred W23 background). The *B'* allele in this stock was a spontaneous derivative of the *B-I* allele obtained from E.H. Coe, Jr. F1 individuals between *B'* and *B-I* Mu were self-pollinated to generate F2 families. F2 families were screened in sand benches for rare darkly-pigmented seedlings resembling *B-I*-like plants among siblings that were essentially green. The *mediator of paramutation1-1* (*Mop1-1*) mutation was isolated from this screen.

[00180] **Genetic Crosses.** In the crosses that follow, a single allele listing indicates homozygosity, whereas heterozygous individuals are indicated with alleles separated by a slash (/). In some instances, the identity of one (dominant) allele is known, but the second allele could be either of two possibilities (e.g. *B'/-*). The term "family" refers to plants grown from kernels on the same ear, all of which share a common tassel parent. To characterize homozygous *Mop1-1* plants relative to heterozygous siblings, families segregating *Mop1-1* were generated as follows: *B' PIR-g Mop1-1* plants were crossed to *B' PIR-g Mop1*, and the resulting F1 plants backcrossed to *B' PIR-g Mop1-1* plants. Unless otherwise noted, all comparisons between *Mop1-1/Mop1-1* and *Mop1/Mop1-1* siblings derive from families generated in this way. One exception is the individuals used to assay the methylation levels of repeated sequences. The sibling individuals compared in this experiment derived from a backcross of *B'/b1*-A188 Pl/*pl-sr R-r/R-g Mop1/Mop1-1* with homozygous *B' PIR-g Mop1-1*.

[00181] To test whether *Mop1-1* affects the paramutant *Pl'* allele, *B' PIR-g Mop1-1* plants were crossed to a *b1*-W23 *Pl' R-r* stock, and F1 individuals were self-pollinated. Additional segregating families were generated by intercrossing light- and dark-anthered siblings. The inheritance of *Pl'* was assayed from four *Mop1-1/Mop1-1* individuals derived directly from the self-pollinated F1 individuals. Additional tests were done using individuals from segregating families derived from *Pl' Mop1/Mop1-1* crossed by *Pl' Mop1-1/Mop1-1*. Tests from these families totaled seven *Pl' Mop1-1/Mop1-1* and three *Pl' Mop1/Mop1-1* individuals. The inheritance of *Pl'* was tested by crosses with a variety of *Pl-Rh* tester stocks.

[00182] To test whether *Mop1-1* influences the expression of other *b1* alleles, *B' PIR-g Mop1-1* plants were crossed to a B-Peru *PIR-g Mop1* stock (inbred W22 background), and

the F1 plants were self-pollinated. Purple kernels were planted (B-Peru/-), and homozygous B-Peru individuals produced ears with 100% purple kernels. All F2 individuals were testcrossed with *B' PlR-g Mop1-1* to determine the *Mop1* genotype. B-bar stocks were derived as follows: B-bar pl-W22 *R-r Mop1* (inbred W22 background) were crossed with *B' PlR-g Mop1-1*; the F1 was self-pollinated; dark progeny (*B'/- Mop1-1/Mop1-1*) were backcrossed with B-bar pl-W22 *R-r Mop1*; progeny of the backcross were self-pollinated; and dark progeny were self-pollinated again. The phenotypes of progeny from the last self-pollinations were used to discern whether *Mop1-1* intensifies B-bar.

[00183] To test whether *Mop1-1* has an effect on the establishment of *b1* paramutation, *Mop1-1* was crossed to *b1*-K55 *PlR-g* to facilitate further genetic analyses in a background independent of *B'*. This *b1*-K55 *Mop1* stock was used to introduce the *Mop1-1* allele into *B-I* stocks to test for an effect of *Mop1-1* on the establishment of *b1* paramutation. Three *B'/B-I Mop1-1/Mop1-1* individuals were tested with one cross each to *B-I*/B-Peru *PlR-g Mop1* stocks and with crosses to *b1* tester stocks (total of four families to either *b1*-K55/*b1*-K55 or *b1*-K55/*b1*-W23 *PlR-g Mop1/Mop1*).

[00184] To test for an effect of *Mop1-1* on the establishment of *pl1* paramutation, *B' Pl-Rh R-r/- Mop1/Mop1-1* was crossed with *B'/- Pl' R-r/- Mop1/Mop1-1*; and darkly-pigmented progeny (*B'/- Pl'/Pl-Rh R-r/- Mop1-1/Mop1-1*) were crossed with *b1*-K55 *Pl-Rh R-r Mop1*. A total of four families representing three *Pl'/Pl-Rh Mop1-1/Mop1-1* individuals were examined.

[00185] To test effects of *Mop1-1* on the establishment of *r1* paramutation, *B' PlR-g Mop1-1* plants were crossed to *R-st* and *R-d*. Intercrosses between the resulting *R-st* and *R-d* progenies produced paramutagenic *R-d R-st* and non-paramutagenic *R-d R-g* heterozygotes for comparison among *Mop1-1/Mop1-1* and *Mop1/-* classes. Because *Mop1-1* does not intensify B-bar pigmentation, one in 13 plants classified as *Mop1/-* (light plant) is expected to be B-bar/B-bar *Mop1-1/Mop1-1*. Reduced paramutation in this genotype would underestimate paramutation in the control population and thus underestimate the ability of *Mop1-1* to inhibit *r1* paramutation. Effects on *r1* paramutation were assayed with testcrosses to W23 *R-g* stocks (null for both aleurone and plant expression of *r1*) followed by measurement of kernel color using a reflectometer (Alleman and Kermicle, 1993). A similar crossing scheme was used to test effects of *Mop1-1* on the paramutable *R-r:std* haplotype. *R-r:std*, also known as standard *R-r*, indicates that this is the specific *R-r* accession originally tested by Brink (1956).

[00186] **Analysis of RNA Levels and Transcription Rates.** RNA levels were assayed by RNase protections using actin or ubiquitin as an internal control. RNA was isolated using

Trizol (Gibco/BRL) according to manufacturer's directions, with the exception that tissue was ground using liquid nitrogen in a mortar and pestle. RNase protections were performed as described in Selinger and Chandler (1999). 5 µg total RNA was used per hybridization. The actin 1 probe (5' half of exon 2), the 315 bp cDNA probe from *b1*, and the 438 bp cDNA probe from c2 were as described previously (Selinger and Chandler, 1999). The ubiquitin probe is a 228 bp BglII fragment from ubi2 (Christensen et al., 1992) which encompasses one of the seven ubiquitin repeats. The *pl1* probe is a 505 bp cDNA fragment which includes exons 1-2 and ends at the BglI site in exon 3 of the *Pl-Rh* allele.

[00187] Transcription rates were determined using in vitro transcription assays on isolated nuclei. Nuclei were isolated by two independent procedures. The first method, using a hexylene glycol-based buffer and percoll for differential density purification, was based primarily upon a maize nuclei isolation protocol (Cone et al., 1993) with modifications incorporated (B. J. Janssen, personal communication) after consulting several additional sources (Spiker et al., 1983; Watson and Thompson, 1986; Paul et al., 1987; Lund et al., 1995). Leaf sheath tissue, harvested from plants approximately two weeks prior to tassel emergence, was ground with a mortar and pestle in liquid nitrogen and suspended in 160 ml nuclear extraction buffer at 4°C (Cone et al., 1993). After filtration through two layers of cheesecloth, 1.6 ml of 25 % Triton X-100 was slowly added to each sample, followed by filtration through a 53 µm nylon sheet. After filtering, samples were centrifuged in a swinging bucket rotor (10 min 4°C 1000g). Nuclei were resuspended in 20 ml working buffer (Cone et al., 1993) modified to contain 0.25% Triton X-100 and layered on top of 90% percoll (Cone et al., 1993) modified to contain 0.25% Triton X-100 and 10 mM MgCl2. Samples were centrifuged in a swinging bucket rotor (20 min. 4°C 4000g). Nuclei were recovered from the top of the 90% percoll, diluted 3-fold in nuclei wash buffer (10 mM PIPES (Piperazine-N,N'-bis[2-ethanesulfonic acid]) pH 7.0, 10 mM MgCl2, 5 mM beta-mercaptoethanol, 20% glycerol) and pelleted (15 min 4°C 2000g). Supernatant was decanted and nuclei were resuspended in resuspension buffer modified to contain 20% glycerol (Hollick and Gordon, 1993).

[00188] In the second method, nuclei from 7-10 grams of sheath and husk tissue from plants at anthesis was prepared using a chromatin isolation protocol (Steinmuller and Apel, 1986) with the following changes: Ground material was suspended in 15 ml isolation buffer (Steinmuller and Apel, 1986) filtered through two layers of cheesecloth followed by a 53 µm nylon sheet. Three centrifugations were performed (15 min -10°C 6000g). Following the first two centrifugations, crude nuclei pellets were resuspended in 15 ml isolation buffer. After the last centrifugation, crude nuclei were resuspended in 2 ml of the resuspension buffer described above.

[00189] Nuclei isolated by either method were repelleted (30 sec RT 5000 rpm using Eppendorf micro-centrifuge) and resuspended in 0.1 ml of resuspension buffer (described above). Reactions and RNA isolations were carried out as described in Hollick and Gordon (1993) with the exception that 100 µCi alpha-32p-CTP (800 Ci/mmole) was used and incubation was at 30°C for 25 minutes. Comparable amounts of labelled RNA for each genotype were used for filter hybridization.

[00190] Slot-blots were prepared with a Minifold II slot-blot system according to manufacturer's directions (Schleicher & Schuell, Keene, NH) using BA85 nitrocellulose filter membrane and 100 ng per slot of purified gene fragments or equivalent amount of linearized plasmid. Ubiquitin, ~975 bp PstI fragment of ubi2 from plasmid ca210 (Christensen et al., 1992), *b1*, ~1970 bp from plasmid pBcDNA (Radicella et al., 1991), and c2, 1450 bp cDNA from plasmid cLC46E (Wienand et al., 1986). Strips of nitrocellulose with the slot-blotted gene fragments were pre-hybridized for 3 hours in 4 ml hybridization solution at 42°C [5X SSPE (1X SSPE = 0.15M NaCl, 0.01M Sodium phosphate, 0.001M EDTA), 0.1% polyvinyl pyrrolidine, 0.1% Ficoll, 50% formamide, 12.5 µg/ml tRNA]. Pre-hybridization solution was replaced with labelled RNA heat-denatured in 2.5 ml hybridization solution. After 60-72 hours at 42°C, strips were briefly rinsed, washed twice for 15 minutes each at 42°C (1 X SSPE, 0.1% SDS (Sodium Dodecyl Sulfate)) and once for 15 minutes at 42°C (0.1 X SSPE, 0.1% SDS).

[00191] The in vitro transcription assay filters and RNase protections were visualized using a Storm 860 PhosphorImager (Molecular Dynamics, Sunnyvale, CA) and signals were quantified using ImageQuaNT software. Background was subtracted from each signal prior to normalizing *b1* and *p1* signals to ubiquitin or actin signals.

[00192] **Analysis of Global DNA Methylation Levels.** Global DNA methylation levels were assayed using methylation sensitive restriction enzymes and DNA blots. DNA was isolated from leaves (Dellaporta et al., 1983). DNA (~4µg) was digested according to manufacturer specifications (New England Biolabs, Bethesda Research Laboratories and Pharmacia), and size-fractionated by electrophoresis in 0.8% agarose gels with 0.5X TBE (0.045M Tris-borate, 0.001M EDTA). The DNA was transferred to charged Hybond N+ (Amersham) membrane with alkaline transfer buffer (0.4M NaOH, 0.6M NaCl). The 45S ribosomal repeat (McMullen et al., 1986; McMullen et al., 1991) and the centromere repeat from sorghum (pSau3a9; Jiang et al., 1996) were radioactively labelled using random hexamer priming (Feinberg and Vogelstein, 1983). Hybridization was performed in a rotating hybridization oven at 65°C with 5% SDS, 50 mM PIPES pH 6.5, 50 mM NaHPO4 pH 7, 1 mM EDTA, 100 mM NaCl and 100 µg/ml salmon sperm DNA. Washes were 2 min 65□C 1X

SSC/0.1% SDS, 30 min 65°C 0.5X SSC/0.1% SDS, 10 min RT 0.1X SSC/0.1% SDS (1X SSC = 0.15 M NaCl, 0.015 M Sodium Citrate). Blots were exposed to a phoshor-imaging screen and visualized with a Storm 860 PhosphorImager (Molecular Dynamics).

[00193] **Identification and Genetic Characterization of mop 1.** Paramutation always occurs when *B'* and *B-I* are heterozygous, and *B'* is extremely stable in subsequent generations (reviewed in Chandler et al., 2000). These characteristics enable a simple genetic screen to isolate mutations affecting paramutation. Furthermore, the phenotypes of *B-I* and *B'* are readily distinguishable even in young seedlings; *B-I* seedlings have intense anthocyanin pigmentation, and *B'* seedlings have little or no anthocyanin pigmentation. A screening population was generated by crossing *B'* plants by *B-I* plants carrying active *Mutator* (Mu) transposable elements. F1 plants were screened for darkly pigmented (*B-I*-like) individuals, which could represent dominant mutations preventing the establishment of paramutation. All F1 individuals (>3500) were lightly pigmented (*B'*), indicating that paramutation had occurred without exception. To identify recessive mutations that may represent failure to maintain the repressed *B'* transcription state, F1 individuals were self-pollinated and F2 families were screened for darkly pigmented (*B-I*-like) seedlings segregating at 1/4 frequency. Darkly anthocyanin pigmented *B-I*-like individuals are referred herein as dark and lightly anthocyanin pigmented *B'*-like individuals are referred herein as light. Several F2 families yielded dark seedlings (25 of 510 families screened). Segregation in subsequent crosses confirmed the presence of a recessive mutation at a single locus in each of these families (data not shown). The high frequency of families segregating dark seedlings was not observed in a similar screen resulting from the cross of a *B'* Mu stock with *B-I*; none of the F2 families yielded dark seedlings (zero of 427 families screened). The high frequency in one set of stocks, but not in the other, suggested that a recessive mutation had been present in some of the *B-I* Mu stocks used in these experiments. Consistent with this hypothesis, genetic complementation tests among plants from the 25 families yielded dark seedlings, confirming that each of these families carried a mutation in the same gene (data not shown). Pedigree analyses demonstrated that this mutation was segregating in the *B-I* Mu stocks. Based on subsequent analyses described below, we have designated the locus identified by this mutation mediator of paramutation1 (*Mop1*), and the mutant allele *Mop1-1*. Recessive *Mop1-1* likely represents a reduced or loss of function allele; individuals heterozygous for *Mop1-1* have *B'* pigment levels, which is defined as wild-type for this genetic background. As these individuals possess the *Mop1* allele normally present in most maize stocks, we will refer to the *Mop1* allele as wild-type.

[00194] ***Mop1-1* Alters the Phenotype of Both *B'* and *PI'*.** The fact that *Mop1-1* was isolated as dark, *B-I*-resembling seedlings among light *B'* siblings indicated that *Mop1-1*

modified the phenotype of *B'* seedlings to resemble a *B-I* phenotype. A similar modification of the *B'* phenotype is observed in mature plants. Figures 3A and 3B show the adult phenotypes of *B' Mop1/Mop1-1* and *B' Mop1-1/Mop1-1* plants. Figures 3B and 3C illustrate the very similar phenotype of *B' Mop1-1/Mop1-1* and *B-I Mop1/Mop1* plants. These results are consistent with the *Mop1* gene having a role in the maintenance of the paramutant *B'* state. Though *B' Mop1-1* plants resemble *B-I* plants, they occasionally show somatic instability, manifest as sectors of *B'*-like pigmentation similar to that seen in Figure 3D. This observation suggests that the *Mop1-1* mutation is unstable and may be caused by a transposable element.

[00195] Does *Mop1-1* also alter the phenotype of the paramutant *Pl'* allele? To address this question, it is necessary to assay the *pl1* expression phenotype independent of changes in *b1* expression, yet to activate the anthocyanin pathway, *pl1* must be co-expressed with a bHLH factor (*b1* or *r1*). The anthers represent a reliable tissue in which to assay *pl1* expression because *r1*, but no *b1* allele, is expressed in the anthers. Fortunately, the *r1* gene expressed in the anthers does not undergo spontaneous paramutation, thus changes in anther pigment reflect changes in *pl1* expression. Our homozygous *B' Mop1-1* stocks lacked *r1* expression in the anthers (homozygous for the null *R-g* allele), therefore we introduced *R-r*, which is expressed in both seeds and anthers. In the presence of *R-r*, *Pl'* gives variegated (light) anthers, whereas *Pl-Rh* gives fully-pigmented (dark) anthers. Light-anthered *B'/b1*-W23 *Pl'/Pl' Mop1/Mop1-1 R-r/R-g* individuals were self-pollinated, and purple kernels (inheriting dominant *R-r*) were planted. Recessive *b1*-W23 produces a green plant when homozygous, but *b1* segregation will not affect anther pigmentation. If *Mop1-1* intensifies *Pl'*, then 1/4 dark-anthered progeny are expected to segregate. Light-anthered individuals, as seen in Figure 3E, and dark-anthered individuals, as seen in Figure 3F, were observed among the F2 progeny at the expected frequency (9 of 47 dark; chi-squared = 0.858, P = 0.35). Three-quarters of these plants were *B'/-* (either *B'/B'* or *B'/b1*-W23), and intensification of the *B'* phenotype in the dark-anthered individuals confirmed they were *Mop1-1/Mop1-1*. This result demonstrated that *Mop1-1* is not specific to *B'* but also affects the paramutant *Pl'* allele.

[00196] ***Mop1-1* Does Not Heritably Alter *B'*, but Can Heritably Alter *Pl'*.** Under some conditions, paramutant alleles can return to the higher expression state characteristic of the paramutable allele. This has been observed for paramutant alleles of *pl1* and *r1*, though not for *b1* (reviewed in Chandler et al., 2000). Genetic crosses were performed to test whether *Mop1-1* heritably alters *B'* to *B-I* or *Pl'* to *Pl-Rh* as diagrammed in Figure 4. When *B' Mop1-1* individuals are crossed with plants null for *b1* and wild-type for *Mop1* (*Mop1*), all progeny are light (*B'*/b *Mop1/Mop1-1*; >400 plants from >20 families) demonstrating the light pigment

phenotype of *B'* is not heritably altered by *Mop1-1*. Furthermore, crosses between *B' Mop1-1* and *B-I Mop1* individuals, both of which are dark, also generate all light progeny [*B'/(B-I)', Mop1/Mop1-1*; >100 plants from 6 families] indicating the *B'* allele transmitted from homozygous *Mop1-1* plants is fully capable of causing paramutation (Figure 4A) in a *Mop1/Mop1-1* nucleus. Thus, *Mop1-1* does not disrupt the inheritance of the *B'* state.

[00197] To test whether the *Pl'* allele in homozygous *Pl' Mop1-1* plants can be heritably altered to *Pl-Rh*, dark-anthered *Pl' Mop1-1/Mop1-1* individuals were crossed with *Pl-Rh Mop1/Mop1* testers (Figure 4B). Progeny of this cross were scored with respect to anther pigmentation, using the anther color scale previously described (Hollick et al., 1995). In this scale, individuals with an Anther Color Score (ACS) of 7 are *Pl-Rh*. Most progeny (~75%) had lightly pigmented anthers, indicating *Pl'* could be transmitted from homozygous *Mop1-1* plants. However, dark-anthered individuals (*Pl-Rh*-like, ACS 7) were sometimes observed as shown in Figure 5. This is in contrast to control crosses in which sibling *Pl' Mop1/Mop1-1* plants were crossed to *Pl-Rh* testers and all progeny had lightly pigmented anthers (Figure 5). Not all *Pl' Mop1-1/Mop1-1* individuals produced dark-anthered progeny. In a total of 12 progeny families, representing testcrosses from 11 different individuals, only six families had some dark-anthered progeny. All six of these families were from homozygous *Pl'/Pl' Mop1-1/Mop1-1* individuals in which one of the *Pl'* alleles was exposed to homozygous *Mop1-1* for two consecutive generations. In contrast, no ACS 7 progeny were observed from any of the four *Pl' Mop1-1/Mop1-1* individuals in which the *Pl'* alleles were exposed to homozygous *Mop1-1* for a single generation. Thus, *Mop1-1* can, but does not always, heritably alter *Pl'* to *Pl-Rh*.

[00198] ***Mop1-1* Increases Transcription of *B'* and Amounts of *Pl'* Transcripts.** To test whether changes in pigment are the result of changes in transcript levels and transcription, as observed with *b1* and *pl1* paramutation (Patterson et al., 1993; Hollick et al., 2000), RNA levels and transcription rate were determined. As depicted in Figure 6, analysis of RNA levels in husk tissue using RNase protection assays reveal a dramatic (46 fold) increase in *b1* RNA in *B' Mop1-1/Mop1-1* relative to *B' Mop1/Mop1-1* siblings. RNase protections also reveal a significant (5.6 fold) increase in *pl1* RNA levels in *Pl' Mop1-1/Mop1-1* relative to *Pl' Mop1/Mop1-1* siblings (Figure 6). These increases in *b1* transcript levels are consistent with (though higher than) the 10-20 fold differences in transcript levels between *B-I* and *B'* (Patterson et al., 1993). Increases in *pl1* transcript levels are also similar to (though less than) those differences seen between *Pl'* and *Pl-Rh* (Hollick et al., 2000).

[00199] To determine if the increased transcript levels of homozygous *B' Mop1-1* plants are associated with increased transcription, in vitro transcription assays were performed on

nuclei isolated from sheath tissue. As shown in Figure 7, these assays reveal that the transcription rate of *b1* is increased in sheaths of *B' Mop1-1/Mop1-1* plants relative to *Mop1/Mop1-1* siblings. The results of three independent pairwise comparisons between sibling *Mop1-1/Mop1-1* and *Mop1/Mop1-1* individuals are shown graphically in Figure 7B. RNase protection assays were done using the same samples to determine the transcript levels in the same sheath tissues. The results of pairwise comparisons of RNA levels for the same six individuals are summarized in Figure 7C. The in vitro transcription assays reveal a 3-4.5 fold increase in transcription rate whereas RNase protection assays reveal a 7-13 fold increase in transcript levels in the same tissues. Similar differences in fold increase (5-8 versus 13-14) of RNA levels versus transcription rate are observed at c2, a gene regulated by *b1* and *pl1* (Figure 7A and data not shown). Overall, the dramatic pigmentation differences between *B' Mop1/Mop1-1* and *B' Mop1-1/Mop1-1* siblings can be explained by increased *b1* transcript levels, which can be at least partially explained by an increase in *b1* transcription rate.

[00200] ***Mop1* Does Not Affect Neutral Alleles.** To determine whether *Mop1-1* is a general modifier of the anthocyanin regulators, or is specific to those alleles that participate in paramutation, we tested the ability of *Mop1-1* to enhance the pigment levels of *b1* and *pl1* alleles that do not participate in paramutation. The B-Peru allele has dark aleurone pigment, has extremely weak plant pigment that is readily distinguishable from *B'*, and does not participate in paramutation (Patterson et al., 1995). As diagrammed in Figure 8, *B' PIR-g Mop1-1* plants were crossed with a B-Peru *PIR-g Mop1* stock, and four F1 individuals were self-pollinated. Only purple F2 kernels were planted to select for homozygous or heterozygous B-Peru. Among these progeny, three classes of plant color phenotypes were observed (Figure 8). As intensification of *B'* plant pigmentation would preclude any conclusions about *Mop1-1* intensification of the lighter B-Peru plant pigmentation, it was essential to compare only homozygous B-Peru individuals, identified by the presence of 100% purple kernels on the ears produced by these individuals. All 24 individuals characterized as having typical B-Peru plant pigment had ears with 100% purple kernels, confirming their *b1* genotype as B-Peru/B-Peru. These plants were testcrossed with *B'/B' Mop1-1/Mop1-1* plants to determine their *Mop1* genotype. If any of these individuals were also homozygous for *Mop1-1*, they would be expected to produce 100% dark progeny (plant phenotype) from the testcrosses with *B' Mop1-1*. Four of the homozygous B-Peru individuals met this expectation, and thus had been homozygous for *Mop1-1*. The fact that their pigment phenotypes were indistinguishable from their B-Peru/B-Peru *Mop1/Mop1-1* or B-Peru/B-Peru *Mop1/Mop1* siblings demonstrates that *Mop1-1* does not intensify B-Peru pigmentation. All plants that had the *B' Mop1-1* or *B' Mop1/-* phenotype (Figure 8) were confirmed to be heterozygous for *B'*.

[00201] In the process of introducing *Mop1-1* into other genetic backgrounds, a stock was generated that contained B-bar and pl-W22, weakly pigmented neutral alleles of *b1* and *pl1*, respectively. Three dark plants, subsequently shown to be B-bar/*B' Mop1-1*/*Mop1-1* pl-W22/pl-W22, were self-pollinated, producing 3/4 dark and 1/4 light progeny (76 dark vs. 25 light). PCR analyses confirmed that the light progeny were homozygous for *B-bar* and the darks all contained at least one *B'* allele. The fact that these light progeny resembled *B-bar Mop1/-* pl-W22 plants demonstrated that *Mop1-1* did not intensify B-bar. As these plants were also homozygous for *pl-W22*, an allele of *pl1* which does not participate in paramutation (Hollick et al., 1995), *Mop1-1* effects on a neutral *pl1* allele could also be examined. All plants had weak *pl-W22* anther color. Together with the results described above with *B-Peru*, these results suggest that the *Mop1-1* intensification of pigment is selective for paramutant alleles.

[00202] ***Mop1-1* Inhibits the Establishment of *b1* Paramutation.** To determine whether *Mop1* is involved in the establishment of *b1* paramutation, we asked whether *B-I* could be paramutated when it encounters *B'* in a homozygous *Mop1-1* nucleus. To address this question, homozygous *B' Mop1-1* individuals were crossed, as diagrammed in Figure 9, with plants heterozygous for *B-I*/*b1*-K55 and *Mop1*/*Mop1-1*. The *b1*-K55 allele is recessive, producing a green plant when homozygous. *B'*/*B-I* and *B'*/*b1*-K55 were differentiated by restriction fragment length polymorphisms between the *B-I* and *b1*-K55 alleles, and each class was crossed with both *B-I*/B-Peru testers (Figure 9) and with *b1* testers (not diagrammed). A feature of the *B-I*/B-Peru cross is that the *B-I* allele is frequently more stable (less prone to spontaneous paramutation to *B'*) when heterozygous with a neutral allele. Furthermore, the purple aleurone pigmentation of B-Peru kernels provides a useful marker for the allele, and the weak B-Peru plant pigmentation is recessive to and readily distinguished from *B-I* or *B'* pigmentation (Radicella et al., 1992; Patterson et al., 1995).

[00203] Among offspring of *B'*/*B-I Mop1-1*/*Mop1-1* plants crossed to *B-I*/B-Peru (*Mop1*/*Mop1*), purple kernels (inheriting the B-Peru allele of the tester) gave rise to plants segregating 50% light (original *B'*) and 50% dark (unaltered *B-I*) individuals, as shown in Table 2. A similar result was obtained among offspring of *B'*/*B-I Mop1-1*/*Mop1-1* plants crossed with *b1* tester stocks (*b1*-K55/*b1*-K55 *Mop1*/*Mop1* or *b1*-K55/*b1*-W23 *Mop1*/*Mop1*). Eighteen of 43 plants were dark or medium-dark and the remaining 25 individuals were light (Table 2). Medium-dark individuals could be explained by spontaneous paramutation of *B-I* to *B'*, or *Pl-Rh* to *Pl'*, which is often observed for these alleles (Coe, 1966; Hollick et al., 1995). Some of these individuals were crossed with appropriate tester stocks, and the phenotypes of progeny were consistent with spontaneous paramutation of *Pl-Rh* causing the reduction in pigment. Control crosses of *B'*/*B-I Mop1*/*Mop1-1* plants to the same *B-I*/B-Peru

and *b1* tester stocks produced all light progeny. These results demonstrate that paramutation is not established in *Mop1-1/Mop1-1* plants.

[00204] Surprisingly, colorless kernels from the same crosses to the *B-I*/B-Peru tester (inheriting the *B-I* allele of the tester) gave fewer than the expected 50% *B-I* plants (Table 2). Many individuals were dark toward the base of the plant, but appeared to lighten progressively during development such that at anthesis, 46 plants were scored as medium or light and only three as dark (*B-I*). Possible reasons for the decreased frequency of *B-I* offspring in this experiment include spontaneous paramutation of the *B-I* allele from the tester, or subtle destabilization of the *B-I* allele transmitted from the *B'/B-I Mop1-1/Mop1-1* plants. These hypotheses are considered more thoroughly in the discussion. Nevertheless, *Mop1-1* is clearly able to inhibit the establishment of paramutation in *B'/B-I Mop1-1/Mop1-1* plants, as dark offspring carrying *B-I* segregate from these individuals. This is in sharp contrast to stocks wildtype for *Mop1* in which paramutation always occurs (Coe, 1966; Patterson et al., 1993; Patterson et al., 1995).

Table 2.
Results of *B'/B-I mop1-1/mop1-1* Test Crosses with several Tester Stocks

Cross		No. of Dark Progeny	No. of Light Progeny	χ^2 (P)[a]
B'/B-I mop1-1/mop1-1 x *b/b Mop1/Mop1*		18[b]	25	1.139 (0.29)
B'/B-I mop1-1/mop1-1 x *B-Peru Mop 1*[c]		26	25	0.0196 (0.89)
B'/B-I mop1-1/mop1-1 x *B-I Mop1*[c]		3	46[b]	37.7 (<0.0001)

[00205] [a] The hypothesis tested is 1:1 segregation.

[00206] [b] Plants were scored as dark or medium-dark. Test crosses demonstrated that medium-darks were *B-I Pl'*.

[00207] [c] *B-Peru Mop1* and *B-I Mop1* represent the different gametes produced by the *B-I/B-Peru* tester.

[00208] [d] Plants were scored as medium (20) or light (26). Many mediums were darker at the base, consistent with spontaneous paramutation.

[00209] ***Mop1-1* May Inhibit the Establishment of *pl1* Paramutation.** We also examined whether *Mop1-1* inhibits the establishment of *pl1* paramutation, though this experiment is complicated by the fact that *Pl'* can occasionally be heritably altered to *Pl-Rh* in homozygous *Mop1-1* plants. To ensure that the allele entering the cross was the paramutagenic *Pl'*, light-anthered *Pl'/Pl' Mop1/Mop1-1* plants (in which *Pl'* is stable and does not change to *Pl-Rh*) were crossed with dark-anthered *Pl-Rh/Pl-Rh Mop1/Mop1-1* plants. Each of these stocks also carried the *B'* allele, which enabled an independent assay of the *Mop1* genotype. Among the progeny, there was perfect cosegregation of dark plant and anther pigmentation (*Mop1-1/Mop1-1*). Three dark-anthered individuals (*Pl'/Pl-Rh*) were crossed with a *b1*-K55 *Pl-Rh* tester to determine whether paramutation occurred (all progeny should have light anthers) or whether the *Pl-Rh* allele could be inherited unaltered (50% dark and 50% light anthered plants). The resulting progeny segregated 39 purple-anthered *Pl-Rh* plants and 36 light-anthered *Pl'* plants. The simplest explanation for this result is that *Mop1-1/Mop1-1* prevented the establishment of paramutation resulting in the segregation of the original *Pl-Rh* and *Pl'* alleles.

[00210] ***Mop1-1* Inhibits the Establishment of *r1* Paramutation.** As discussed above, the phenomenology of *r1* paramutation is very different from that of *b1*. We asked whether *Mop1-1* is able to affect paramutation at *r1*. Our *B' Mop1-1* stocks contained the *R-g* allele of *r1*, which does not participate in paramutation (Kermicle et al., 1995). These *Mop1-1 R-g* stocks were crossed with stocks wildtype for *mop1* (*Mop1*) and containing *r1* haplotypes that participate in paramutation. The term haplotype is used for complexes composed of multiple *r1* genes. The *r1* haplotypes used included paramutagenic *R-st*ippled (*R-st*) and two paramutable haplotypes, standard *R-r* and *R-d*:Catspaw (*R-d*). The F1's between *R-st* and *Mop1-1* (*R-st/R-g*; *Mop1/Mop1-1*) were intercrossed with F1's of the paramutable haplotypes (*R-d/R-g* or *R-r/R-g*; *Mop1/Mop1-1*). Thus, the paramutagenic and paramutable haplotypes were combined in homozygous *Mop1-1* mutant progeny, and in siblings wildtype for *Mop1* (*Mop1/-*). Fifty percent of intercross progeny inherited the paramutable haplotype (*R-d* or *R-r*), and among these, four genotypes of interest segregate as shown for *R-d* in Figure 10A. The presence of *B'* allows the *Mop1* genotype to be easily monitored. Testcrosses of *Mop1/-* (either *Mop1/Mop1* or *Mop1/Mop1-1*) individuals in which *R-d* was heterozygous with neutral *R-g* provide a baseline for wild-type kernel pigmentation. Testcrosses of *R-d/R-g* plants homozygous for *Mop1-1* did not differ detectably from wildtype (*Mop1/-*), as shown in Figure

10B. Paramutation occurred as expected in the *R-st/R-d Mop1/-* individuals, manifest as reduced pigment in the *R-d* progeny (Figure 10B). However, siblings homozygous for *Mop1-1* showed no reduction of *R-d* expression (Figure 10B). Thus, in individuals homozygous for the *Mop1-1* mutation, paramutable *R-d* exits the cross as if it had been heterozygous with a neutral allele rather than with paramutagenic *R-st*. Similar results were obtained with the paramutable standard *R-r* (not shown). This demonstrates that *Mop1-1* is able to inhibit the establishment of *r1* paramutation.

[00211] **Mutations in *Mop1* Correlate with Pleiotropic Effects.** As shown in Table 3 and Figure 11B, 11C and 11D, plants homozygous for *B' Mop1-1* can show direct or indirect developmental abnomalities. The range of effects seen include delayed flowering or reduced stature relative to wild-type siblings, spindly and sometimes barren stalks, and in some instances, aberrant development resulting in feminized tassels (such as that observed in tasselseed mutants--Irish et al., 1994). We reproducibly see differences in flowering time, whereas other abnormalities appear stochastically. There may be an environmental effect on the frequency with which these developmental abnormalities occur, as the frequency has been highest in our Hawaii nursery during the past two winters. Two alternative hypotheses could explain the correlation of these effects with *Mop1-1*. The effects could be caused either by other mutations that are linked to *Mop1-1*, or by the *Mop1-1* mutation itself. Recently, a second allele of *Mop1* was isolated from a similar screen looking for modification of *Pl'* instead of *B'* and using ethyl methanesulfonate (EMS) as the mutagen (Example 3). This independent allele (*Mop1-2EMS*) provides independent data with which to judge the two alternative hypotheses. *B'/- Mop1-2EMS/Mop1-2EMS* and *B'/- Mop1-1/Mop1-2EMS* individuals show the same suite of pleiotropic phenotypes as homozygous *Mop1-1* (Table 3). This strongly suggests that these negative pleiotropic effects are the result of the mutant *Mop1* locus, rather than the result of linked mutations.

Table 3. Developmental Phenotypes[a] Associated with *Mop1* Mutants

	Seg			Phenotypes *Mop1*		*Mop1*				
Family	Exp	Obs	χ^2(P)	N[b]	O	N	STS	WTS	BT	R[c]
Mop1-1 segregating	1:1	37:39	0.05 (0.82)	36	1[d]	13	15	3	3	5
Mop1-2EMS segregating	3:1	75:15	3.33 (0.07)	74	1[e]	6	2	1[f]	4	2
Mop1-1/Mop1-2EMS segregating	1:1	13:15	0.14 (0.71)	13	-	6	3	1	2	3

[00212] [a] Phenotypes are as follows: *Mop1*, lightly pigmented plant; *Mop1*, darkly pigmented plant; N, normal; O, other; STS, strong tasselseed; WTS, weak tasselseed; BT, barrenized tassel; R, runty or scrawny plant.

[00213] [b] The number of individuals represent progeny from four, five, and two plants for the *Mop1-1*, mop 1-2EMS, and *Mop1-1/Mop1-2EMS* segregating crosses, respectively.

[00214] [c] One of five, one of two, and one of three plants also possessed a barrenized tassel, and one of five plants possessed a feminized tassel, which failed to emerge (see Figure 11C).

[00215] [d] This plant was slightly deformed with a twisted stalk.

[00216] [e] This plant was diseased.

[00217] [f] This plant was shorter than were sibling plants and did not produce an ear.

[00218] ***Mop1-1* Does Not Affect Global Methylation Levels.** Although no differences in DNA methylation are observed in comparisons of the paramutagenic and paramutable alleles of *b1* and *pl1* (within the coding and ~15 kb of the respective flanking regions, Patterson et al., 1993; Hollick et al., 2000), paramutation of *R-r* correlates with DNA methylation in the transcribed region (Walker, 1998). In other systems, changes in DNA methylation often correlate with differences in transcription, thus one model is that *Mop1* encodes a protein that influences global DNA methylation levels such as a DNA methyltransferase (Finnegan et al., 1996) or ddm1 (decrease in DNA methylation, Vongs et al., 1993). Under this model, one could hypothesize that methylation differences occur at *b1* and *pl1*, but these changes occur outside of the regions examined. We tested whether global DNA methylation levels are affected by *Mop1-1*. DNA blots with methylation sensitive restriction enzymes were prepared to compare homozygous *B' Mop1-1* individuals, wild-type siblings (*B' Mop1/Mop1-1*), and *B'* stocks (*Mop1/Mop1*). These blots were hybridized with repeated sequences of the 45S ribosomal region (McMullen et al., 1986) as well as a

repeated sequence found at centromeres (Jiang et al., 1996). As shown in Figures 12A-12B, no differences in DNA methylation were detected among the *Mop1* genotypes.

[00219] ***Mop1* and Paramutuation.** Our results demonstrate that *Mop1* plays a central role in paramutation; the *Mop1-1* mutation disrupts the maintenance and establishment of paramutation at multiple maize loci, but has no effect on alleles that do not participate in paramutation. The pleiotropic developmental phenotypes that stochastically occur in some families segregating *Mop1* mutations suggest that *Mop1* may have additional regulatory functions beyond paramutation.

[00220] **Paramutation at *b1*, *pl1* and *r1* Shares a Common Mechanism.** There are unique aspects to the behavior of *b1*, *pl1* and *r1* alleles participating in paramutation (reviewed in Chandler et al., 2000). *r1* paramutation involves structurally distinct haplotypes of a complex or repeated nature. In contrast, *B'* and *Pl'* are spontaneous derivatives of *B-I* and *Pl-Rh*, respectively. Furthermore, increased methylation is readily apparent in *R-r'* relative to *R-r* (Walker, 1998), whereas such differences have not been observed for *b1* and *pl1* (Patterson et al., 1993). We have demonstrated that *Mop1-1* disrupts the maintenance of *B'* and *Pl'* expression, the heritability of *Pl'*, and the establishment of *b1* and *r1* paramutation. These results provide compelling evidence that, despite several phenomenological differences, all three loci use a common molecular factor for paramutation, strongly suggesting that a common mechanism underlies paramutation at these three loci.

[00221] **A Role for *MOP1* in the Establishment and Maintenance of the Paramutant State.** Our results indicate *MOP1* function is clearly involved in all three phases of gene silencing; establishment, maintenance, and heritability (reviewed in Loo and Rine, 1995). When paramutable and paramutagenic alleles of *b1*, *pl1* or *r1* are heterozygous, paramutation always occurs (reviewed in Kermicle, 1996; Chandler et al., 2000), yet in nuclei homozygous for *Mop1-1*, paramutation was not established at *b1*, *r1* or *pl1*. When *Pl-Rh* was introduced to *Pl'* in homozygous *Mop1-1* plants, *Pl-Rh* appeared to be transmitted unaltered as 50% dark-anthered (*Pl-Rh*) and 50% light-anthered (*Pl'*) plants segregated. This interpretation is complicated by the fact that homozygous *Pl' Mop1-1* plants can sometimes produce dark-anthered *Pl-Rh* progeny. However, it seems unlikely that the 50% dark-anthered plants resulted from full establishment of paramutation and subsequent reversion of half the *Pl'* alleles to *Pl-Rh*. The establishment experiment used *Pl'* alleles exposed to homozygous *Mop1-1* for a single generation, a condition in which no *Pl-Rh* progeny were observed in other experiments. Thus, the simplest explanation for the 50% segregation observed in this experiment is that *Mop1-1* inhibits the establishment of *pl1* paramutation.

[00222] One possibility is that *MOP1* mediates or facilitates the interaction between the paramutagenic and paramutable alleles, such that loss of function disrupts that interaction. *MOP1* may be necessary for the paramutagenic properties of *B'*, *Pl'* or *R-st*, such that loss of function makes these alleles more similar to neutral or paramutable alleles. Perhaps consistent with this, the *B'* allele is modified by *Mop1-1* such that, in homozygous *Mop1-1*, *B'* resembles *B-I* with respect to phenotype, transcription, and the inability to paramutate another *B-I* allele. The appearance of phenotypically *B'* sectors in *B' Mop1-1* plants further suggests that *MOP1* can function throughout development to reduce the expression of *B'* such that regaining *MOP1* function in a somatic sector re-establishes the reduced expression state.

[00223] The effect of *Mop1-1* on *B'* provides a very clear demarcation between two of the phases of gene silencing, maintenance and inheritance, as described by Loo and Rine (1995). Though functional *MOP1* is required to maintain the reduced transcription state of *B'*, loss of *MOP1* function is not sufficient to disrupt the inheritance of the *B'* state as it is faithfully and completely restored upon recovering *MOP1* function. In fact, we know of no condition in which *B'* is heritably changed to *B-I*. We have speculated this is because *B'* represents the default transcription state specified by the DNA sequence (Patterson et al., 1993).

[00224] **Allele Interactions May Influence the Stability of Epigenetic States.** Allelic interactions may account for our observation that *B-I* segregates at different frequencies from *B-I/B' Mop1-1/Mop1-1* plants depending on what it is crossed with. In crosses to the neutral alleles B-Peru and *b1* (*b1*-K55 or *b1*-W23), *B'/B-I Mop1-1/Mop1-1* plants transmitted 50% dark individuals, completely consistent with the failure to establish paramutation in the *B'/B-I Mop1-1/Mop1-1* plants. Interestingly, fewer than the expected 50% *B-I* individuals were observed segregating from *B'/B-I Mop1-1/Mop1-1* plants in crosses to *B-I*. Observation of *B-I* progeny at the anticipated 50% frequency would require three conditions: inheritance of an unaltered *B-I* allele from the *B-I*/B-Peru tester parent, inheritance of an unaltered *B-I* allele from *B'/B-I Mop1-1/Mop1-1* individuals, and stability of both *B-I* alleles in the homozygous *B-I* progeny individuals. Homozygous *B-I* is generally less stable than *B-I*/B-Peru. The developmentally progressive reduction in pigment of many (presumed *B-I/B-I*) progeny plants appeared similar to, and thus could be explained by, the progression sometimes observed during spontaneous paramutation of *B-I* to *B'*. This result could also be caused by a higher than usual rate of spontaneous paramutation of the *B-I* allele segregating from the *B-I*/B-Peru tester. Alternatively, the *B-I* allele segregating from *B'/B-I Mop1-1/Mop1-1* may be subtly destabilized. This subtle destabilization could be exacerbated in the next generation by homozygosity with a second *B-I* allele, whereas it could be overcome in the next generation by heterozygosity with a neutral allele such as B-Peru or *b1*.

[00225] **A Model for *MOP1* Function and Paramutation.** Numerous facets of *b1*, *r1* and *pl1* paramutation are consistent with chromatin structural changes, rather than DNA sequence changes, underlying paramutation. The absence of detectable DNA sequence differences between *B'* and *B-I*, the multiple levels of *Pl'* expression, and the instability of the paramutant *R-r'* and *Pl'* states fit well with a chromatin model. We propose that *MOP1* functions as a chromatin remodeling protein. As observed for some chromatin related proteins, individuals mutant for *Mop1* can show developmental phenotypes (Grossniklaus et al., 1998; Eshed et al., 1999; Kakutani et al., 1999; Ogas et al., 1999). We hypothesize that *MOP1* is involved in the assembly of a repressive chromatin structure at *b1*, *pl1* and *r1* upon paramutation. This repressive structure could be analogous to Polycomb group (PcG) complexes assembled at Polycomb Response Elements (PRE, Pirrotta, 1998; Hollick et al., 1997).

[00226] The distinct expression states of *B-I* vs. *B'* and *Pl-Rh* vs. *Pl'*, as well as the spontaneous and directed alteration of the paramutable alleles, could be explained by this model. The alleles that participate in paramutation are hypothesized to possess PRE-like elements. In the high expression state (*B-I* or *Pl-Rh*), the elements are not efficiently recognized for PcG-like assembly. We postulate that the chromatin structure can spontaneously change allowing access of PcG-like proteins for assembly, resulting in the *B'* and *Pl'* states. The fully assembled state of the *B'* and *Pl'* alleles, when together in a nucleus with the unassembled state, would induce assembly on the paramutable allele (Patterson et al., 1993; Hollick et al., 1997).

[00227] Variations on this model can explain differences in stability between the *Pl'* vs. the *B'* states, and the different effect *Mop1-1* has on the heritability of this state. Once assembled in *B'*, the PcG-like complex would remain quite stable due to multiple, strong interactions between members of the complex and DNA binding sites. When *MOP1* function is lacking in *B' Mop1-1* homozygotes, several remaining members of the complex stay associated with the PRE-like binding sites, such that restoration of *MOP1* function quickly re-establishes the repressive structure, just as some residual proteins are thought to mark PREs for rapid reassembly in Drosophila (Pirrotta, 1998). Divergence or fewer binding sites at *pl1* could result in a less stable complex relative to *b1*. Loss of *MOP1* function in *Pl' Mop1-1* homozygotes, combined with reduced or compromised binding sites, could destabilize the complex such that residual association of the complex with the PRE-like element is reduced in some nuclei resulting in the *Pl-Rh* state.

[00228] **Mutants Affecting Epigenetic Silencing or Allelic Interactions.** Active research into many trans-sensing phenomena involves identifying mutations that disrupt the

interaction. As paramutation and other trans-sensing interactions share many features, these screens could identify *Mop1* homologues. Our results suggest that *Mop1* is unlikely to represent a DNA methyltransferase or ddm1-orthologue, because the *Mop1-1* mutation does not alter methylation levels of repetitive centromeric sequences or the 45S ribosomal repeated sequences. The ddm1 locus was identified based upon its effect on methylation levels of repetitive sequences in Arabidopsis, such as the ribosomal genes and centromeric repeats (Vongs et al., 1993). The ddm1 locus has been cloned and it shares similarity with SNF2, a yeast protein involved in chromatin remodeling (Jeddeloh et al., 1999). Interestingly, a related protein in humans has also been shown to decrease global DNA methylation when mutated (Gibbons et al., 2000). Similarly, decreased activity of the DNA methyltransferase of Arabidopsis achieved by antisense expression of a MET1 transgene reduces the methylation levels of repeated sequences (Finnegan et al., 1996).

[00229] Several mutations altering the transcriptional silencing of plant transgenes have been identified (Furner et al., 1998; Mittelsten Scheid et al., 1998; Amedeo et al., 2000). Some of these mutations turn out to be allelic to ddm1 (Mittelsten Scheid et al., 1998; Jeddeloh et al., 1999), whereas one encodes a novel protein (Amedeo et al., 2000). Several mutations have been identified that disrupt cosuppression or post-transcriptional gene silencing (Elmayan et al., 1998), In is not yet clear whether transcriptional and post-transcriptional gene silenciing are mechanistically related (reviewed in Meyer and Saedler, 1996). A screen for mutations affecting transvection at the yellow locus of Drosophila identified exclusively cis-acting mutations at yellow (Morris et al., 1999). Numerous mutations in Drosophila have been isolated that affect position-effect variegation, identifying proteins associated with and involved in chromatin structure (Sass and Henikoff, 1998; Wakimoto, 1998; Wallrath, 1998; Cryderman et al., 1999).

Example 2

Mutations that Affect Paramutation in Maize Also Reverse Mu Transposon Methylation

[00230] **Introduction.** Very little is known about the mechanism of paramutation. At *r1*, there is a correlation between the methylation state of some parts of the gene and its expression state (Walker 1998). Also at *r1*, there is a relationship between the degree of paramutagenicity of an allele and the number of repeats of the coding region and flanking DNA (Kermicle et al.1995). As a transposon (*doppia*) is present in the regulatory region of several paramutable alleles of *r1*, it has been suggested that this transposable element is causally related to paramutation. However, this relationship remains correlative, and there are paramutagenic alleles of *r1*, such as *R-marbled*, that do not carry this transposon

(Panavas et al. 1999). Further, despite the fact that the promoter region of the paramutable *B-I* allele is littered with transposable elements, no correlation between methylation of these elements and transcription level has been observed at *b1* (Patterson et al. 1993), or *pl1* (Hollick et al. 2000). Thus, there is no consistent correlation to date between paramutation and transposable element methylation within or nearby a gene.

[00231] Recently, a mutation, *Mop1-1*, has been isolated which prevents paramutation at *b1*, *r1*, and *pl1* (Dorweiler et al. 2000). When this mutation is homozygous, the normally low expressing allele of *b1*, *B'*, expresses at the level of the high expressing allele, *B-I*. This results in the dark purple plant color characteristic of the *B-I* allele as opposed to the lighter and sporadic purple color characteristic of the *B'* allele. Further, when *B'* is heterozygous with *B-I* in a *Mop1-1* homozygous background paramutation is prevented, with both the *B'* and *B-I* phenotypes transmitted upon out crossing. In the absence of the *Mop1-1* mutation only the *B'* phenotype is transmitted.

[00232] Although in *Mop1-1* homozygotes *B'* expresses at the level of *B-I* and it cannot paramutate *B-I*, it is not heritably altered to *B-I*, because when out crossed to generate a heterozygote (*Mop1/Mop1-1*) *B'* is observed. These results suggest that the *Mop1* gene product is involved with the process of paramutation, but it does not alter the heritable state of *B'*. The *Mop1-1* mutation also prevents the establishment of paramutation at the p1l and *r1* loci, indicating that the *Mop1* gene product is a general regulator of paramutation (Dorweiler et al. 2000).

[00233] Herein we show that mutations in *Mop1* can prevent and reverse Mu element methylation. Despite this reversal of methylation, we do not see reactivation of Mu element transposition in these genetic backgrounds, suggesting that the Mu inactive state is not reversible by *Mop1* mutations. Because the *Mop1* mutation affects multiple loci, each of which has very different promoter sequences, we hypothesize that this mutation is operating on chromatin configuration, rather than specific sequences.

MATERIALS AND METHODS:

[00234] **Genetic Crosses.** In the crosses that follow, a single allele listing indicates homozygosity, whereas heterozygous individuals are indicated with alleles separated by a slash (/). Following maize genetic nomenclature, recessive alleles are indicated by lower case and dominant alleles are indicated by capitalization of the first letter. Unless indicated otherwise all stocks carry functional alleles of all the anthocyanin biosynthetic genes. The

alleles of the regulatory genes *r1*, *b1*, and *pl1* that are in each stock are indicated for each cross.

[00235] **Generation of a *Mop1-1* homozygote.** The *Mop1-1* homozygous plant used in crosses with *MuKiller* was generated as follows: *B' PIR-g* (recessive allele of *r1* with no pigment and no paramutation activity) *Mop1-1* plants were crossed to *B' PIR-g Mop1*, and the resulting F1 plants were back crossed to *B' PIR-g Mop1-1* plants. Homozygous *Mop1-1* mutants are darkly pigmented in leaf sheaths, husks and tassel branches and glumes, while the *Mop1/Mop1-1* heterozygotes are lightly pigmented in the same tissues (Dorweiler et al. 2000). As this stock was derived from an active *Mutator* stock, it carried multiple *MuDR* elements and hypomethylated *MuDR* and *Mu1* elements as evidenced by DNA blotting (not shown).

[00236] **Generation of a plant carrying *MuKiller*.** To detect *MuDR* activity we used *a1-mum2*. This mutation contains a *Mu1* insertion in the 5' promoter region of the a1 anthocyanin biosynthesis gene (O'Reilly et al. 1985). In the presence of active *MuDR* elements excisions of *Mu1* can be scored as somatic purple sectors in the kernels. *MuKiller*, a dominant factor present in some lines but not in our minimal *Mutator* line, is competent to silence a single *MuDR* element (Lisch and Freeling 1994). A plant carrying a single *MuDR* element on chromosome 2L (Chomet et al. 1991) with the genotype *B' pl-sr* (allele of *pl1* that confers sun-red anthers and husks) *R-g* (*r1* allele that confers purple seed) *a1-mum2 MuDR/-* was crossed to a line carrying *MuKiller* (*MuK*) and no active *MuDR* elements (*B' pl-sr R-g a1-mum2 MuK/+*). The resulting plants were scored for the presence of newly silenced *MuDR* elements as indicated by the reduced excision frequency of the *Mu1* element from *a1-mum2* and methylation of *Mu1* elements in the presence of *MuDR*. Of nine individuals examined in this family, three carried at least one full-length *MuDR* element and several methylated *Mu1* elements, consistent with the epigenetic silencing of *MuDR* elements by the *MuK* activity segregating in this family. The first ear of one such plant was used in the cross with a *Mop1-1/Mop1-1* homozygote as described below. The second ear was crossed to a plant lacking *MuDR* elements and homozygous for the *a1-mum2* reporter (Chomet et al. 1991). None of the kernels resulting from this test cross showed evidence of excisions of *Mu1* from *a1-mum2*, confirming the silencing of *MuDR* in this family.

[00237] **Crossing *Mop1-1* to *MuKiller*.** A single *B' PIR-g* A1 *Mop1-1* plant was crossed to a plant carrying a silenced *MuDR* element (as described above). The resulting progeny were self-fertilized, yielding *Mop1-1* homozygotes, *Mop1-1/Mop1* heterozygotes and *Mop1* homozygotes. To generate a second generation of *Mop1-1* homozygous plants, *B' Mop1-1* homozygous plants resulting from the above cross were identified by their dark plant pigment

and were crossed to *Mop1-1/Mop1* siblings and the resulting plants were scored for the *Mop1-1* phenotype. To test for reversals of Mu element methylation, *Mop1/Mop1-1* progeny from that cross, which carried methylated Mu elements, were self fertilized to generate *Mop1-1* homozygous mutants that had been heterozygous and methylated in the previous generation.

[00238] **Isolation of *Mop1-1* stocks lacking *MuDR*.** Plants carrying *Mop1-1* in the absence of full length *MuDR* were isolated from the same genetic background as those carrying full length *MuDR* elements by screening for the loss of *MuDR* using DNA blots. The absence of *MuDR* was determined by digestion with SacI, XbaI, and EcoRI + HindIII. Diagnostic fragments for full length *MuDR* elements were absent in these lines.

[00239] **Isolation of stocks carrying silenced *MuDR* elements.** Plants carrying *MuDR* at the original position from which it was first cloned (Chomet et al. 1991) were crossed to plants carrying *MuKiller*. Progeny that carried both *MuDR* and methylation of HinfI sites in *Mu1*elements, consistent with the activity of *MuKiller*, were self-fertilized to generate stocks that were homozygous for silenced *MuDR*. The progeny kernels, all of which lacked excisions of *Mu1* from the reporter *a1-mum2* allele, were screened by DNA gel blot for the presence of homozygous *MuDR* elements. These plants were again self-fertilized to generate stocks carrying homozygous silenced *MuDR* elements.

[00240] Generation of seeds carrying a single silenced *MuDR* element that were also homozygous for *Mop1-2EMS*. Plants carrying homozygous silenced *MuDR* elements (*a1-mum2*; *MuDR*, *R-g*) were crossed to plants that were A1, *R-g*, *Mop1-2EMS* (an EMS-induced allele of *Mop1*) and that lacked full-length *MuDR* elements. The progeny plants were self-fertilized, and the resulting families were screened for the presence of spotted kernels. If *Mop1-2EMS* reactivated silenced *MuDR* elements, then only the resulting kernels that were homozygous for *a1-mum2* (1/4), carried *R-g/R-g* or *R-g/R-g* (and not *r1* mottled) (1/2), homozygous for *Mop1-2EMS* (1/4) and carrying *MuDR**(3/4) would be expected to be spotted. Thus, the expected frequency of spotted kernels was 2.34 % (1/4 x 1/2 x 1/4 x 3/4). In addition to self-fertilization, some plants were crossed to plants that were homozygous for *Mop1-1*, *a1-mum2*, and *R-g*. The expected frequency of spotted kernels in the families generated from these crosses was 6.5 % (1/2 *a1-mum2/a1-mum2* x 1/2 *R-g/R-g* or *R-g/R-g* x 1/2 *MuDR* x 1/2 *Mop1/Mop1*).

[00241] **DNA preparation and genomic blotting.** DNA preparation and genomic blotting were performed as previously described (Dorweiler et al. 2000). A plasmid of *Mu1* was as previously described (Talbert et al. 1989). To generate an internal probe for *Mu1*, the

plasmid was digested with AvaI and BstEII, and the internal fragment was gel isolated. An internal fragment of *MuDR* bounded by EcoRI and BamHI was as described (Chomet et al. 1991). The *b1* upstream probe used to generate the data shown in Figures 14 and 16 was previously described in (Patterson et al. 1993) and (Patterson et al. 1995). As a control for partial digestion of DNA, blots were reprobed with a KpnI fragment of the a1 gene spanning a region of the coding sequence adjacent to (but not including) the *Mu1* insertion in this gene (O'Reilly et al. 1985). As a control for the blot with the SacI digest in Figure 13, the blot was probed with a single copy PstI fragment flanking but not including the *MuDR* insertion on chromosome 2L (not shown) (Lisch et al. 1995). That same probe was used to identify individuals containing that specific *MuDR* insertion in *MuDR* reactivation experiments.

RESULTS

[00242] **The *Mop1-1*mutation prevents the dominant methylation of Mu elements.** The *Mop1-1* mutation was originally isolated in a *Mutator* active line (Dorweiler et al. 2000) with multiple copies of both *MuDR* and *Mu1*, all of which were hypomethylated (data not shown). To examine the interaction between the *Mop1-1* mutation and methylation of the *Mutator* system, it was necessary to inactivate the active *MuDR* elements in the *Mop1-1* line. To accomplish this, a *Mop1-1* homozygote was crossed to a plant from a family carrying *MuKiller* (*MuK*) a factor that dominantly inactivates the *Mutator* system (Lisch and Freeling 1994). Details of these crosses are provided in Materials and methods within this example.

[00243] Several plants resulting from the cross between the *Mop1-1* mutant and the plant carrying *MuK* were self-fertilized, and progeny were visually scored for the *Mop1-1* phenotype (dark purple plants). The methylation status of both the autonomous *MuDR* elements and the non-autonomous *Mu1* and related *Mu1.7* elements was determined using DNA blots. One such family is shown in Figure 13. In this family, 21 progeny were examined (Table 4, family 1162-3x). Four of the 21 plants exhibited a strong *Mop1-1*/*Mop1-1* phenotype (lanes 1-4). As the parent of this family was *B'*, the dark purple color of these plants was diagnostic for the presence of the *Mop1-1* mutation. In addition to their dark pigment these plants were significantly shorter than their wild-type siblings, and none of them produced ears, consistent with previous observations of pleiotropic effects of this mutation (Dorweiler et al. 2000). To determine the effect of *Mop1-1* on methylation of *Mu1* elements due to *MuK*, DNA from these plants was digested with HinfI and probed with an internal *Mu1* fragment (Figure 13A). As a digestion control, the blot of the HinfI digest was probed with a fragment of the a1 gene flanked by HinfI sites that are not normally methylated (Figure 13B). To assay for changes in methylation of *MuDR* termini, the same samples were digested with SacI and probed with an internal *MuDR* fragment (Figure 13C). Because both of these enzymes are methylation

sensitive, a reduction or elimination of the expected internal fragment is indicative of methylation of these sites.

[00244] Of the *Mop1-1/Mop1* or *Mop1/Mop1* siblings (lanes 5-21), half (9/17) had methylated *Mu1* elements (Figure 13A). The SacI digest revealed that although these particular plants did have the fragment diagnostic for at least one full length *MuDR* element, it was reduced in intensity and that reduction was accompanied by the appearance of additional, larger fragments (Figure 13C). This observation is consistent with the methylation of sites within both *MuDR* and *Mu1* TIRs seen previously in plants undergoing epigenetic silencing (Chandler and Walbot 1986, Martienssen and Baron 1994, Lisch and Freeling 1994), and is presumably due to the activity of *MuK* in this family. In contrast, none of the four *Mop1-1* homozygotes had methylated *Mu1* or *MuDR* elements.

[00245] Additional individuals from two other similarly derived families (Table 4, families 1181-1x and 1162-2x) were examined. A total of 41 progeny from self fertilizations of *Mop1-1/Mop1* heterozygotes from this first generation were examined by both phenotype and DNA gel blot, including 10 *Mop1-1* homozygotes and 31 of their wild-type siblings. The frequency of methylation of *Mop1/Mop1* and *Mop1/Mop1-1* individuals depended on the methylation status of the parent. In the two families generated from the self-fertilization of plants that had hypomethylated *Mu1* elements (1162-3x and 1181-1x), a total of 13 of the 24 plants with the wild-type phenotype (*Mop1/Mop1* or *Mop1-1/Mop1*) had methylated *Mu1* elements. In the family generated from a parent that already had methylated *Mu1* elements (1162-2x), all seven of seven wild-type progeny had methylated *Mu1* elements. In contrast, the *Mu1* elements in all 10 of the *Mop1-1/Mop1-1* homozygotes from the three families carried hypomethylated *Mu1* elements. Given the frequency of methylated Mu elements in their wild-type siblings, we can expect with 99.9% confidence that at least one of the mutants would have had methylated *Mu1* elements if *Mop1-1* were not affecting methylation.

[00246] To test the heritability of this phenomenon, *Mop1-1* homozygotes from the family shown in Figure 13 were crossed to three different Mu inactive hypermethylated wild-type siblings. One family produced no *Mop1-1* plants, suggesting that the inactive parent lacked the *Mop1-1* mutation. Two other families (1510 and 1511) segregated for the *Mop1-1* phenotype in the expected ratio (14/29 and 5/17 dark progeny, respectively). An analysis of family 1511 is shown in Figure 14. Family 1511 was derived from a cross between the two siblings shown in Figure 13 (that in lane 4 by that in lane 15). In both of these families, all of the resulting *Mop1-1/Mop1-1* plants carried hypomethylated *Mu1* elements. In contrast, none of the wild-type *Mop1/Mop1-1* plants carried hypomethylated *Mu1* elements (Table 4).

Table 4

Methylation of *Mu* in Families Segregating for the *mop1-1* Mutation

	male: *Mu-active, mop1-1/mop1-1*			self: *Mu-active; mop1-1/Mop1*		
	female: *Mu-inactive, mop1-1/Mop1*					
	Male	Female		Self	Self	
	1511	1510	Total	1162-3x	1181-1x	Total
mop [a]	5	14	19	4	3	7
mop methylated	0	0	0	0	0	0
non-*mop* [b]	12	15	27	17	7	24
non-*mop* methylated	12	15	27	9	4	13
total plants assayed	17	29	46	21	10	31

self:	Mu-inactive; *mop1-1/Mop1*				Grand total	
	Self	Self	Self	Self		
	1510-9x	1510-1x	1162-2x	1511-6x	total	
Mop	3	1	3	3	10	36
Mop methylated	0	0	0	0	0	0
non-*mop*	7	13	7	11	38	89
non-*mop* methylated	7	13	7	11	38	78
total plants assayed	10	14	10	14	48	125

[00247] [a] *mop1/mop1* homozygotes showing the dark purple plant phenotype

[00248] [b] *mop1/Mop1* or*Mop1/Mop1*, not showing the dark purple plant phenotype

[00249] **The *Mop1-1*mutation reverses the effects of previous methylation of the Mu elements.** To confirm that the *Mop1-1* mutation could reverse previously established *Mu1* element methylation, several plants that were heterozygous for *Mop1-1*and that carried methylated *Mu1* elements (Figure 14) were self-fertilized. The resulting families were scored for both the *Mop1-1* phenotype (dark purple pigment) and methylation of *Mu1* elements (families 1510-1x, 1510-9x, and 1511-6x, Table 4). In the figure, one individual from family 1511 (Panel A, lane 6) was self-fertilized to give rise to family 1511-6x (Panel D). Consistent with the segregation of a single recessive mutation, roughly one quarter (7/38) of all of these progeny had the *Mop1-1* phenotype (Table 4). All 7 of the *Mop1-1/Mop1-1* homozygotes examined had hypomethylated *Mu1* elements relative to their wild-type siblings, all of which carried methylated *Mu1* elements. Thus, the *Mop1-1* mutation reverses previously established methylation of *Mu1* elements.

[00250] **The *Mop1-1* mutation reverses *Mu1* methylation in the absence of full-length *MuDR* elements.** Normally, there is a very tight correlation between hypomethylation of *Mu1* elements and the presence of intact, functional *MuDR* elements, suggesting that the *MuDR* transposase is required to prevent a default methylation pathway that targets Mu elements (Chandler and Hardeman 1992). The *Mop1-1* homozygous plants provide the first exception to this rule. Although *Mop1-1* was derived from a *Mutator* line, we have identified several lineages that lack intact, functional *MuDR* elements. That is, they lack diagnostic fragments when digested with SacI (Figure 15), XbaI, or with an EcoRI, HindIII double-digest (data not shown). Based on this observation, we conclude that these lines do not carry intact, functional *MuDR* elements. Despite this, we find that the *Mop1-1* plants in these lines still carry hypomethylated *Mu1* elements. We examined two families in more detail, one of which is shown in Figure 15. In these families, which were the products of the self-fertilization of plants heterozygous for *Mop1-1*, hypomethylation of *Mu1* cosegregated with the *Mop1-1* phenotype. Including the individuals shown in Figure 15, a total of eight *Mop1-1* mutant and eight wild-type siblings were examined. The *Mu1* elements of all of the *Mop1-1* plants were hypomethylated relative to the *Mu1* elements of their wild type siblings.

[00251] **A second allele of *Mop1* also hypomethylates *Mu1*.** To eliminate the possibility that there was a factor modifying *Mu1* methylation that was only coincidentally

linked to *Mop1-1*, we also examined a family segregating a different allele of *Mop1*, *Mop1-2EMS*. This family was derived from a non-*Mutator* stock that had been EMS mutagenized (Hollick and Chandler 2001). DNA from six *Mop1-2EMS* mutants and six *Mop1/Mop1-2EMS* siblings were digested with HinfI and probed with *Mu1*. As with *Mop1-1*, the *Mu1* elements in the mutant plants were significantly less methylated than their wild-type siblings (data not shown).

[00252] **The *Mop1-1* mutation does not reverse methylation of sites immediately upstream of the *b1* locus.** Given that the *Mop1-1* mutation reverses *Mu1* methylation, we wanted to determine whether or not it affects methylation of sequences elsewhere in the genome. We were particularly interested in examining sequences around *B'*, as mutations in *Mop1* increase transcription of *B'* (Dorweiler et al. 2000). There are a number of sites upstream of the *B'* and *B-I* transcription unit (these alleles share identical restriction maps) that have been previously assayed for methylation status (Patterson et al. 1993, Patterson et al. 1995) (GenBank, accession no. X70790 S48060). No differences in methylation had been observed between *B-I* and *B'* plants, suggesting that, unlike the situation at the *r1* locus, methylation in the promoter proximal and 5' untranslated region is not correlated with the 10-20 fold difference in transcription. The methylation status of various restriction sites upstream of *B'* and *B-I* is shown in Figure 16. We observed no difference between *B' Mop1-1*, *B' Mop1/Mop1-1*, and *B-I Mop1* plants at the HinfI site 1.8 kb upstream of the transcriptional start site (Figure 14, panel C) or between *Mop1-1* homozygotes and heterozygotes at the ApaI, SalI, or PvuII sites (Figure 16). Each of the last three digests was performed in combination with BamHI. The complete absence of the expected 12 kb BamHI fragment in each of the double digests suggests that at least one of the sites recognized by the methyl-sensitive enzyme in each double digest was digested to completion. Thus, partial digestion of any specific site was due to methylation, rather than incomplete digestion. Interestingly, these sites are all within transposable elements, suggesting that methylation of transposons in the promoter proximal region of *B'* is not affected by *Mop1-1*. These elements include a MITE element immediately upstream of the start of transcription and the Muse element (a distant relative of *MuDR*; V. Chandler, D. Selinger and M. Stam, personal observation). Together, these data suggest that *Mop1-1* is not a global regulator of transposon methylation.

[00253] **Progeny of plants homozygous for the *mop1-1* mutation for multiple generations can show *Mu* element somatic excision :** To determine if reactivation could occur in subsequent generations, one family segregating for both the *mop1-2EMS* mutation and a single silenced *MuDR* element was planted, subjected to DNA gel blot analysis, and out-crossed. Nine plants from this family were examined. Four of the nine plants contained hypomethylated *Mu1* elements, consistent with good penetrance of this aspect of the *mop1-*

2EMS phenotype. Using a probe flanking the single *MuDR* element in this family, the presence of *MuDR* was confirmed in five of the nine plants. Two plants carried both hypomethylated *Mu1* elements (*mop1-2EMS* homozygotes) and the silent *MuDR* element. One of these plants died. The other was self-fertilized and out crossed to a plant that was *mop1-1*/Mop1 and *a1-mum2*. None of the 288 progeny kernels from the self-fertilization or the 241 kernels from the outcross showed excision of *Mu1* from *a1-mum2*.

[00254] To explore this issue further, we used a more sensitive assay, reactivation of somatic excision of a reporter *Mu1* element. Plants that lacked full-length *MuDR* elements and that were homozygous for *Mop1-2EMS* (the EMS-induced allele of *Mop1*) were crossed to plants homozygous for a single *MuDR* element that had been silenced using *MuKiller* (see Materials and Methods within this Example for details of the cross). The resulting plants were either self-fertilized, or crossed to plants homozygous for *a1-mum2*, and *Mop1-2EMS*. The resulting ears were screened for the appearance of spotted kernels. If *Mop1-2EMS* could activate the silenced *MuDR* elements, 2.34% of the progeny kernels from the self fertilizations, or 6.25% of the test cross progeny kernels would be expected to have excisions. Twenty-two families constisting of a total of 6,324 progeny kernels resulting from self-fertilization were examined. The expected number of spotted kernels in these families was 148 (see Materials and Methods within this Example for calculation). Three families with a total of 710 kernels generated from out crosses to *a1-mum2* testers were also examined. If *MuDR* elements were reactivated by *Mop1-2EMS*, we would have expected to see 45 spotted kernels in these families. None were observed in any of these families, indicating no reactivation of the silenced *MuDR* element.

[00255] To determine if reactivation could occur in a subsequent generation, one family segregating for both the *Mop1-2EMS* mutation and a single silenced *MuDR* element was planted, subjected to DNA gel blot analysis, and out-crossed. Nine plants from this family were examined. Four of the nine plants contained hypomethylated *Mu1* elements, consistent with good penetrance of this aspect of the *Mop1-2EMS* phenotype. Using a probe flanking the single *MuDR* element in this family, the presence of *MuDR* was confirmed in five of the nine plants (data not shown). Two plants carried both hypomethylated *Mu1* elements (*Mop1-2EMS* homozygotes) and the previously silent *MuDR* element. One of these plants died. The other was self-fertilized and out crossed to a plant that was *Mop1-1/Mop1* and *a1-mum2*. None of the 288 progeny kernels from the self-fertilization or the 241 kernels from the outcross showed excision of *Mu1* from *a1-mum2*. These results indicate that even two generations of exposure to *Mop1* mutations is insufficient to reactivate the silenced, but hypomethylated *MuDR* element in this genetic background.

[00256] To determine whether or not *MuK*, which may have been in this lineage, was contributing to the continued repression of *Mu1* somatic excision, despite the presence of *mop1-2EMS*, an additional generation was examined. Even if *MuK* had been homozygous in the original plant, two generations of out crossing would result in half of the progeny lacking *MuK*. Therefore, fifty pale seeds of the 241 seeds from the first outcross described above were planted. DNA from the resulting 45 plants was isolated and subjected to DNA gel blot analysis, which confirmed that half of the plants (23) carried the *MuDR* element at position p1 *MuDR*(p1). Slightly less than one half (18) were homozygous for *mop1-2EMS* mutant. DNA blot analyses demonstrated that the *Mu1* elements in all of the *mop1-2EMS* homozygous plants were hypomethylated relative to their wild type siblings. None of the wild type plants carried any hypomethylated *Mu1* elements. One fifth of the plants (8) were homozygous for both *mop1-2EMS* and had *MuDR* at the p1 position. These plants were outcrossed again to plants that were heterozygous for *mop1-2EMS* and homozygous for *a1-mum2* and *R-g*. Importantly, some of the progeny kernels of the plants that had been homozygous *mop1-2EMS* homozygous and that carried *MuDR*(p1) showed *Mu1* excision from the *a1-mum2* allele. Of six ears from plants with this genotype, four ears had some spotted kernels (a total of 29 of 642 kernels were spotted). The excision frequency in these kernels was variable, ranging from only a few excisions per kernel to a frequency typical for a single active *MuDR* element. In contrast, in 13 ears generated from plants that were heterozygous for *mop1-2EMS* and that carried *MuDR*(p1) there were no spotted kernels out of a total of 3309 pale kernels. Similarly, in 9 ears generated from plants that were homozygous for *mop1-2EMS* but which lacked *MuDR*(p1) there were no spotted kernels out of 1900 pale kernels, and in five ears generated from plants that were heterozygous for *mop1-2EMS* and that lacked *MuDR*(p1), there were no spotted kernels out of 989 pale kernels. The presence of spotted kernels only in the plants homozygous for *mop1-2EMS* and carrying *MuDR*(p1), strongly suggests that contamination by pollen carrying active MuDR is an unlikely explanation for the spotted kernels. Based on these data, we conclude that in the absence of *MuK*, and after two generations of exposure of *mop1-2EMS*, this mutation can cause reactivation of silenced *MuDR* elements.

Example 3

Isolation of *Mop1-2EMS*, *rmr1-1*, *rmr1-2* and *rmr2-1* Mutants

[00257] **Genetic Stocks.** All stocks contain dominant, functional alleles for all genes required for the production of anthocyanin pigments in seedling and anther tissues unless otherwise noted. Pistillate parents used for the mutagenesis experiment had the following *pl1* and *r1* genotypes: *Pl'*; *R-r* or *R-r*/*R-g*. Staminate parents for the mutagenesis experiment

were originally obtained from the Maize Cooperation Stock Center (Urbana, IL; accession no. 611A: *Pl-Rh* sm1; *R-r*). The salmon silks 1 locus is 10 cM distal to *pl1*. The recessive sm1 allele confers salmon colored silks when homozygous and thus provides a linked morphological marker to *Pl-Rh*. The *Pl'* testers used for initial crosses with putative mutants were obtained via spontaneous paramutation of *Pl-Rh* (Hollick et al. 1995). A632 (pl-A632; *R-r*), A619 (pl-A619; *R-g*) and W22 (pl-W22; *R-g*) inbred material was obtained from the USDA North Central Plant Introduction Station, (Ames, IA). Additional W22 stocks (pl-W22; *R-r*:standard) were provided by Jerry Kermicle (Univ. of Wisconsin, Madison). Five *Pl-Rh/Pl-Rh* tester stocks of different genetic backgrounds were used in crosses to determine whether or not mutant plants carried paramutagenic *Pl'* alleles. Material for *pl1* RNA measurements was produced by crossing plants homozygous for the given EMS-derived mutation by sibling plants heterozygous for the same mutations. Additional details of the specific genetic stocks used for these experiments are available upon request.

[00258] **Pollen Mutagenesis.** Pollen pooled from multiple tassels was treated with EMS and applied to silks according to Neuffer and Coe (1978). Pistillate parents were derived from two related *Pl'/Pl'* families. Both families together yielded 345 ears with an average of 49 kernels per ear. Germination frequency was approximately 77% for the first 7500 M1 seed planted. A total of 9000 M1 seedlings were screened for dominant mutations affecting pigment production. Seedlings were grown in potting flats at an approximate density of 100 per sq ft. Approximately 1000 M1 plants were grown to maturity and self-pollinations of M1 plants generated 495 M2 families.

[00259] Seedling Screens. M2 families of 30 seeds each were germinated in unheated sand benches and grown under high-intensity lighting (1660 □E/ m2 sec using a 1:1 mixture of sodium vapor and metal halide lamps). Visible seedling phenotypes (Table 5) were noted between 14 and 18 days post-imbibition.

TABLE 5

EMS-derived M_2 seedling mutations

Mutant Class	Number of EMS-Derived Mutations	Frequency of EMS-Derived Mutations	Frequency of EMS-Derived Mutations (Neuffer)
Chlorophyll deficient Albino	15	.09	.13
Lemon white	21	.12	.037
Yellow green	14	.08	.015

TABLE 5

EMS-derived M_2 seedling mutations

Mutant Class	Number of EMS-Derived Mutations	Frequency of EMS-Derived Mutations	Frequency of EMS-Derived Mutations (Neuffer)
Pale green	16	.09	.015
Variable mutants			
Piebald	1	.006	.03
Striped	12	.076	.09
Premature chlorosis	7	.04	NR
Cross-banded	3	.02	NR
Seedling lethals	37	.21	.12
Glossy	8	.05	.026
Dwarf	6	.04	NR
Others	63	.36	.29

[00260] The number and frequency of visible mutations identified in the current M2 screen are compared with the frequency of similar mutations identified from an earlier EMS pollen mutagenesis (Neuffer 1978). NR: None Reported.

[00261] **Genetic Crosses and Stock Syntheses.** Hand pollinations were used for all genetic crosses. Material for the sm1, rm*r1* cosegregation test was derived by crossing a single plant heterozygous for ems136 and homozygous sm1 to a plant homozygous for ems235 but heterozygous for the recessive sm1 allele. The following syntheses and analyses were used to generate material to test the effects of the EMS-derived mutations on neutral *pl1* alleles. Plants heterozygous for a given mutation (*Pl'* anthers) were crossed to both the A632 inbred line and a W22 line (obtained from J. Kermicle, Univ. of Wisconsin, Madison) containing the weakly expressed *pl1* alleles pl-A632 and pl-W22 respectively. The pl-A632, pl-W22, and *Pl-Rh* alleles are all distinct based on RFLP analyses (J. Hollick,

unpublished). F1 plants were either self pollinated (A632 material) or backcrossed to plants homozygous for the given mutation (W22 material). F2 (A632 material) and BC1 (W22 material) progeny were grown to maturity. The *pl1* genotypes of all plants in the F2 and BC1 families were determined using RFLP gel blot analysis as previously described (Hollick et al. 1995). Anther phenotypes of all homozygous pl-A632 F2 plants were photographed for later comparison. All homozygous pl-A632 F2 plants were crossed to plants heterozygous for the given EMS-induced mutation to determine whether the F2 plants were also homozygous for the given EMS-induced mutation. Two F2 plants were identified that were homozygous pl-A632 by RFLP analysis and homozygous for *rmr1-1* by testcross analysis and five F2 plants were identified that were homozygous pl-A632 by RFLP analysis and homozygous for *rmr2-1* by testcross analysis. Examination of the anther photographs did not indicate any pigment differences between A632 inbred anthers or any F2 pl-A632 plants regardless of whether or not they carried the EMS-induced mutations. The BC1 plants that were *Pl'*/pl-W22 were self pollinated and also crossed to plants heterozygous for a given EMS-induced mutation to identify BC1 plants that were also homozygous for EMS-induced mutations. Anther pigmentation of pl-W22/pl-W22; *rmr1-1*/*rmr1-1* and pl-W22/pl-W22; *rmr2-1*/*rmr2-1* plants was weak and not obviously different from the anther pigmentation of heterozygous siblings or grandparental W22 plants.

[00262] **RNA Measurements.** RNA isolations from anther tissues and RNase-protection assays were performed as described in Example 1.

[00263] **Pollen mutagenesis generated new maize mutations.** The number of mutable loci affecting paramutation-based gene silencing is unknown. To identify such loci, we performed chemical mutagenesis using ethyl methanesulfonate (EMS) to produce a high frequency of new maize mutations. M1 seed was obtained by applying EMS-treated pollen from *Pl-Rh*/*Pl-Rh* plants to the silks of receptive *Pl'*/*Pl'* ears. A total of 495 M2 families were subsequently generated by self pollination of M1 plants to screen for recessive mutations. Small M2 families (30 seeds each) were grown for 14-18 days and then visually examined for germination frequencies and unusual morphological phenotypes. Table 5 outlines the frequency of mutant phenotypes identified. Observed mutation frequencies are similar with previous EMS-pollen mutageneses (Neuffer 1978) indicating that our chemical mutagenesis was highly efficient in producing new maize mutations.

[00264] **Dominant Mutations Affecting *Pl'* Expression Were Not Found.** Because the primary leaf sheath of *Pl'*/*Pl-Rh* seedlings is normally weakly pigmented (Hollick et al. 1995), dominant EMS-induced mutations that either release *Pl'* from a repressed expression state or prohibit the establishment of paramutation might be expected to confer fully colored M1

seedling phenotypes. Sixteen out of 9000 M1 seedlings examined were fully colored. However, no flowering plants from these 16 fully colored seedlings had fully colored, *P1-Rh*-like, anthers. This result indicates that our seedling screen for dominant mutations affecting anther pigmentation has an approximate 0.2% false-positive rate. In addition, none of the approximately 1000 M1 plants grown to maturity had a *P1-Rh* phenotype. Thus, no dominant mutations affecting either the establishment of *p11* paramutation or the maintenance of *P1'* repression were identified out of 10,000 M1 plants tested.

[00265] **Genetic screens identified recessive mutations affecting seedling and anther pigmentation.** Recessive mutations that release *P1'* from a repressed expression state might also be expected to confer fully colored seedling phenotypes (Figure 17). Our M2 screen, identified five families (nos. 60, 96, 136, 235, 240) that segregated fully colored seedlings to weakly colored seedlings in the following ratios (60, 4:23; 96, 1:29; 136, 6:24; 235, 3:25; 240, 8:16). In four of five families, fully colored seedlings gave rise to mature plants with fully pigmented *P1-Rh*-like anthers (Figure 18A). Material from family 60 was dropped from further analyses because plants from fully colored seedlings in family 60 did not have fully pigmented anthers.

[00266] Genetic segregation tests indicated that the *P1-Rh*-like phenotypes seen in three of the M2 families (nos. 96, 136, and 235) were heritable as single locus recessive traits. Plants with fully colored anthers derived from the three M2 families were first crossed with *P1'/P1'* plants to complement the putative recessive mutations. All F1 plants from these crosses had a clear *P1'* phenotype (family 96, 35/35; family 136, 36/36; family 235, 36/36) indicating that the putative mutations affecting seedling and anther color are recessive. F1 plants from families 96, 136 and 235 were self pollinated to recover the fully colored seedling and anther trait. In all three cases, fully colored anther phenotypes were recovered at roughly a 1:3 ratio in F2 families (Table 6) consistent with the interpretation that *P1-Rh*-like phenotypes are due to single locus recessive mutations. As expected, subsequent crosses between mutant plants and heterozygous siblings gave rise to families in which the mutant and normal phenotypes approximated a 1:1 ratio (family 96, 231:272; family 136, 100:88; family 235, 111:125).

TABLE 6

Inheritance of EMS-induced mutations

EMS Allele	*P1-Rh* Anthers	*P1'* Anthers	Frequency of Plants with *P1-Rh* Anthers
ems96	8	33	0.24

ems136	9	52	0.17
ems235	46	170	0.27

[00267] EMS-induced factors affecting *Pl'* are inherited as single gene recessive mutations. The number and frequency of plants with fully colored anthers are indicated for the following number of F2 families: *ems96*, 3 families; *ems136*, 4 families; *ems235*, 6 families.

[00268] **Complementation Tests Define Three Loci.** Genetic complementation was tested in pair-wise combinations among the four EMS-induced mutations and also with the recessive a3-Styles allele (Styles and Coe 1986) and the recessive *Mop1-1* allele (Dorweiler et al. 2000). Specific EMS-induced mutations were designated according to the family in which they were identified: ems96, ems136, ems235 and ems240. For each mutation, plants heterozygous for a given mutation (*Pl'* anthers or lightly colored plant in the case of a3-Styles heterozygotes) were individually crossed by plants homozygous for a different allele (*Pl-Rh*-like anthers or darkly colored plants in the case of a3-Styles homozygotes). Anther pigment phenotypes of progeny from these crosses were quantified (Table 7) on a 1-7 graded Anther Color Score (ACS) where ACS 7 corresponds to the fully colored *Pl-Rh* phenotype (Hollick et al. 1995). Based on these results, mutations ems136 and ems235 fail to complement and thus define a single locus, ems240 is allelic to *Mop1-1*, and ems96 defines a third locus. Further description of the ems240 allele, designated *Mop1-2EMS*, is provided in Example 1.

TABLE 7

Complementation Tests

Alleles Tested	Ears Examined	Number of Plants with Given Anther Color Score						
		1	2	3	4	5	6	7
ems96								
a3-Styles	4	1	11	98	2	0	0	0
mop1-1	1	6	6	1	0	0	0	0
ems136	3	8	26	12	1	0	0	0
ems235	5	10	37	27	1	0	0	0
ems240	4	9	46	19	4	0	0	0
ems136								
a3-Styles	2	1	7	20	1	1	0	0
mop1-1	1	9	6	1	0	0	0	0
ems235	3	8	14	2	0	0	0	26
ems235								
a3-Styles	2	0	5	28	3	2	0	0
mop1-1	1	3	11	0	0	0	0	0
ems240	2	0	8	11	4	1	0	0

TABLE 7

Complementation Tests

Alleles Tested	Ears Examined	Number of Plants with Given Anther Color Score						
		1	2	3	4	5	6	7
ems240								
mop1-1	1	0	2	5	0	0	0	11

[00269] Complementation tests identify three unique loci. For each allele listed in bold text, the underlying series of alleles were tested for complementation. The number of ears sampled for each test are indicated along with the total numbers of mature plants with a given Anther Color Score derived from the seeds off those ears.

[00270] **EMS-Derived Mutations Allow Increased Expression of *pl1* RNA.** We used RNase-protection experiments to compare *pl1* RNA levels from anthers of *Pl'/Pl'* plants either homozyogus or heterozygous for the ems136, ems235, or ems96 alleles. Using actin RNA as a control, *pl1* RNA levels were 26, 14, and nine fold greater in homozygous ems136, ems235 and ems96 mutants versus heterozygous siblings, respectively (Figures 18B and 18C). These increases in *pl1* RNA due to the ems136 mutation occur in the absence of any changes in the transcription rate of the *pl1* gene. In vitro transcription reactions using isolated husk nuclei (see example 1), demonstrate that both *pl1* and *b1* transcription rates remain unchanged while a1 transcription is increased 4-fold in homozygous ems136 plants (5 independent experiments) (Figure 21). However, the *pl1* RNA increases due to the ems235 mutation occur with concomitant increases in *pl1* transcription rates. In vitro transcription reactions using isolated husk nuclei (see example 1), demonstrate that *pl1* transcription rates are increased 2.5-fold in homozygous ems235 plants (5 independent experiments) (Figure 22). The *b1* transcription rate remains unchanged and the a1 transcription rate is increased 5-fold. These results indicate that the normal functions of these genes identified by mutations are required to maintain repression of *pl1* RNA accumulation. The transcription assays imply that there are both transcriptional and post-transcriptional regulatory mechanisms involved in maintaining the repression of paramutant *Pl'*.

[00271] **Genes Affecting Repression Of *Pl'* Encode Trans-Acting Factors.** Both *Mop1* and the locus defined by the ems96 allele genetically map to chromosome 2 whereas the *pl1* locus is found on chromosome 6. Alleles that define the other locus (ems136 and ems235) failed to cosegregate with a genetic marker closely linked to the *pl1* locus. The salmon silks 1 (sm1) locus, located 10 cM distal to *pl1*, normally conditions yellow maize silks but plants that are homozygous for the recessive sm1 allele (sm1) have salmon-colored silks. Genetic crosses were used to ask whether the fully-colored anther phenotype found in homozygous mutant plants cosegregated with the recessive sm1 allele. A total of 6/15 (40%) segregant plants with fully-colored anthers were sm1/sm1 and 9/21 (42%) plants with weakly-colored anthers were sm1/sm1. The lack of strong cosegregation between the fully-colored anther phenotype and recessive alleles of the sm1 locus indicates that the locus defined by the ems136 and ems235 alleles is distinct from the *pl1* locus. Thus all three loci identified in our genetic screen define trans-acting genetic factors affecting *pl1* RNA accumulation in *Pl'/Pl'* plants.

[00272] **All Three Loci Identified By Mutation Encode Factors Required To Maintain Heritable Repression Of *Pl'*.** The fully-colored phenotypes of seedlings and anthers together with the increases in *pl1* RNA seen when the EMS-derived mutations are homozygous suggested that *Pl'* may have changed to a *Pl-Rh* state. Plants homozygous for *Mop1-1* sometimes show heritable changes of *Pl'* to *Pl-Rh*. To test mutations at the other two loci, mutant plants with fully-colored anthers were crossed to a series of *Pl-Rh/Pl-Rh* testers and the anther phenotypes of the progeny were quantified. If *Pl'* heritably changes to *Pl-Rh* in plants homozygous for the ems136, ems235, or ems96 alleles then fully-colored *Pl-Rh* phenotypes should be found in the resulting progeny. If *Pl'* does not heritably change to *Pl-Rh*, then only *Pl'* phenotypes would be found in the progeny (Hollick et al. 1995). Results of these crosses (Table 8) indicate that while *Pl'* can be transmitted, *Pl'* often changes to a meiotically-heritable *Pl-Rh* state in plants homozygous for the ems136, ems235 or ems96 alleles. We have designated these new loci "rmr" for required to maintain repression to reflect the necessity of rmr functions for maintaining *Pl'* in a mitotically- and meiotically-heritable repressed state. The ems136 and ems235 alleles together define the rm*r1* locus and are designated *rmr1-1* and *rmr1-2* respectively. The ems96 allele defines the rmr2 locus and is designated *rmr2-1*.

[00273] *Pl'* most frequently changed to a non-paramutagenic (*Pl-Rh*) state in plants homozygous for either the *rmr1-1* or *rmr1-2* alleles (Table 8). Approx. 70% of crosses between *Pl-Rh/Pl-Rh* testers and plants homozygous for either the *rmr1-1* or *rmr1-2* allele produced at least one progeny plant having a *Pl-Rh* anther phenotype compared to only 22% of similar testcrosses with plants homozygous for the *rmr2-1* allele. This difference may

partially relate to observations showing that different *Pl-Rh*/*Pl-Rh* testers themselves have different frequencies of spontaneous paramutation; *Pl-Rh* can spontaneously change to *Pl'* in the absence of *Pl'* (Hollick et al. 1995). To control for such differences, *Pl-Rh*/*Pl-Rh* pollen collected from a single plant was used to pollinate *rmr1-1* and *rmr2-1* homozygous plants. Both sets of crosses produced at least one progeny plant with a *Pl-Rh* phenotype indicating that both *rmr1-1* and *rmr2-1* mutations can allow meiotically-heritable derepression of *Pl'*. However, proportionally more *Pl-Rh*-like progeny were derived from crosses made with homozygous *rmr1-1* plants (Table 9). In addition, progeny plants had higher Anther Color Scores from the *rmr1-1* crosses relative to the *rmr2-1* crosses suggesting that *Pl'* alleles are, in general, less paramutagenic when transmitted through plants homozygous for *rmr1-1* as compared to *rmr2-1* (Tables 8 and 9).

[00274] Given that *Pl'* could sometimes change to a meiotically-heritable *Pl-Rh* state in either homozygous *rmr1-1* and *rmr2-1* plants, we asked whether such newly changed *Pl'* alleles, formally designated Pl('), were distinguishable from a naive *Pl-Rh* allele. It was possible that Pl(') might retain residual paramutagenic activity relative to *Pl-Rh*. A cosegregation test using linked sm1 markers was used to show that a Pl(') allele transmitted from a either a *rmr1-1* or *rmr2-1* homozygote was indistinguishable from naive *Pl-Rh* in terms of its paramutagenicity (Figure 19). Thus, both RM*R1* and RMR2 functions contribute to the meiotically heritable maintenance of the paramutagenic *Pl'* state. In the absence of RM*R1* or RMR2 function, *Pl'* always has a somatic expression phenotype indistinguishable from *Pl-Rh* but this does not ensure that *Pl'* always changes to a meiotically-heritable *Pl-Rh* state.

TABLE 8

Pl' changes to *Pl-Rh*

Pl'/Pl'; ems/ems X Pl-Rh/Pl-Rh and *Pl-Rh/Pl-Rh X Pl'/Pl'; ems/ems*									
Allele Tested	Ears Examined	Number of Plants with Given Anther Color Score							Frequency of Pl-Rh types
		1	2	3	4	5	6	7	
ems136	21 (15/21)	40	113	100	38	26	22	61	0.15
ems235	9 (6/9)	4	33	46	26	8	13	17	0.12
ems96	40 (9/40)	267	390	84	46	13	0	80	0.09

[00275] Genetic crosses listed at the top of the table were used to determine whether or not *Pl'* alleles could change to a non-paramutagenic *Pl-Rh* state in plants that were homozygous for the given EMS-derived alleles. The total number of ears sampled is given along with the fraction of ears that gave rise to plants with fully-colored anthers. The total numbers of progeny with a given Anther Color Score are listed along with the frequency of progeny having a *Pl-Rh* phenotype (ACS 7).

TABLE 9

Pl' changes to *Pl-Rh* using a single pollen source

Pl'/Pl'; rmr/rmr X Pl-Rh/Pl-Rh									
Allele Tested	Ears Examined	Number of Plants with Given Anther Color Score							Frequency of Pl-Rh Types
		1	2	3	4	5	6	7	
rmr1-1	4 (2/4)	4	21	10	24	7	0	9	0.12

rmr2-1	4 (1/4)	24	20	5	5	2	0	1	0.02

[00276] The *Pl'* state can change to *Pl-Rh* in plants homozygous for mutations in *rmr* loci. Genetic crosses using a shared source of *Pl-Rh/Pl-Rh* pollen were made as indicated at the top of the table with female parents homozygous for the given *rmr* alleles. The total number of ears sampled is given along with the fraction of ears that gave rise to plants with fully-colored anthers. The total numbers of progeny with a given Anther Color Score are listed along with the frequency of progeny with a *Pl-Rh* phenotype (ACS 7).

[00277] **rmr Mutations Do Not Affect The Expression Of Other *pl1* Alleles.** We addressed the possibility that rmr functions generally affect all *pl1* alleles by combining neutral *pl1* alleles with either the *rmr1-1* or *rmr2-1* alleles and examining the anther phenotypes. The neutral pl-A632 and pl-W22 alleles confer weak, sunlight-dependent, pigmentation to the anthers. Anthers of plants homozygous for the pl-A632 or pl-W22 alleles had visibly identical levels of pigmentation regardless of whether or not the plants were homozygous for a rmr mutation (Figure 20) implying that RM*R1* and RMR2 functions specifically affect the expression of paramutant *Pl'* alleles.

[00278] **Heritable Changes Of *Pl'* To *Pl-Rh* In rm*r1* Mutants Are Unaffected By Mode Of Sexual Transmission.** Given that male and female gametes are often differentially imprinted in plants (Kermicle and Alleman 1980; Vielle-Calzada et al. 2000) and they arise at different times and locations during development, we wondered whether meiotically-heritable changes of *Pl'* to *Pl-Rh* occurred with equal frequencies in the two separate somatic cell lineages or whether there were gametophyte-specific effects. This possibility was addressed using reciprocal crosses between *Pl-Rh/Pl-Rh* testers and plants homozygous for the *rmr1-1* allele. Pollen from *Pl-Rh/Pl-Rh* testers was placed on receptive silks of *Pl'/Pl'*; *rmr1-1/rmr1-1* plants and vice versa. Plants derived from seven reciprocal crosses were grown to maturity and the Anther Color Scores determined (Table 10). When all the data are combined, the same frequency of *Pl-Rh* progeny was observed when *Pl'* was transmitted through either female or male gametes. When each reciprocal cross was examined, three sets of families had very similar frequencies. Frequencies for the remaining four sets of families varied 1.5 to two fold but there was no sex-specific trend. The frequencies of *Pl'* to *Pl-Rh* changes observed between individual *rmr1-1/rmr1-1* plants could vary as much as six fold but the frequencies observed within each pair of reciprocal crosses varied no more than two fold. Thus, the frequency of heritable *Pl'* to *Pl-Rh* changes in *rmr1-1* homozygotes appears to be

intrinsic to each individual sporophyte and is not differentially affected by female versus male gametophyte development.

TABLE 10

Transmission of *Pl-Rh* from *rmr1-1* plants

Reciprocal Parent Crosses	Number of Progeny Plants with Given Anther Color Scores							Frequency of *Pl-Rh* Types
	1	2	3	4	5	6	7	
98-720-10 X 98-649-1	0	5	3	3	2	0	4	0.24
98-649-1 X 98-720-10	1	1	2	4	1	2	6	0.35
98-720-19 X 98-645-1	0	2	9	2	3	1	3	0.15
98-645-1 X 98-720-19	0	1	2	5	5	1	6	0.3
98-720-22 X 98-648-3	0	0	1	2	2	2	14	0.67
98-648-3 X 98-720-22	0	2	1	0	3	1	12	0.63
98-721-4 X 98-648-1	0	3	2	4	0	0	9	0.5
98-648-1 X 98-721-4	0	2	0	6	3	2	6	0.32
98-721-12 X 98-646-4	0	0	3	0	1	1	13	0.72
98-646-4 X 98-721-12	0	0	0	1	2	1	11	0.73
98-721-17 X 98-645-2	0	2	4	2	0	5	9	0.41
98-645-2 X 98-721-17	0	0	0	1	2	1	18	0.82
98-721-18 X 98-648-4	0	1	0	1	0	0	17	0.89
98-648-4 X 98-721-18	0	0	0	1	2	0	9	0.75

TABLE 10

Transmission of *Pl-Rh* from *rmr1-1* plants

Reciprocal Parent Crosses	Number of Progeny Plants with Given Anther Color Scores							Frequency of *Pl-Rh* Types
	1	2	3	4	5	6	7	
Total for *rmr1-1* females	0	13	22	18	8	9	87	0.55
Total for *rmr1-1* males	1	6	5	18	20	10	82	0.58

[00279] **rm*r1* and rmr2 Mutants Do Not Appear To Affect Plant Development.** Although mutations in *Mop1* can lead to a wide range of developmentally abnormal phenotypes (Example 1) we have not observed grossly abnormal phenotypes in homozygous rm*r1* and rmr2 mutant plants. We began this series of observations by first generating F1 individuals between A632 inbred plants and plants homozygous for either *rmr1-1*, *rmr1-2* or *rmr2-1* and then examining the F2 homozygous mutants derived from self pollination. This strategy was adopted to reduce the number of other unlinked EMS-induced mutations that might potentially affect plant morphology. Of the 200 rmr F2 plants segregating for each rmr mutation, all plants that had a fully-colored anther phenotype were otherwise similar in stature, morphology, and flowering time to sibling plants. F2 families generated using the rmr mutations and both the A619 and W22 inbred lines gave identical results.

[00280] Further generations of inbreeding did not produce abnormal plants. Several rmr F2 plants (A632 background) with fully-colored anthers that had a *Pl-Rh/Pl-Rh* RFLP genotype were self pollinated and also outcrossed to *Pl-Rh/Pl-Rh* testers. All F3 plants from the self pollinations had fully-colored anthers but were otherwise similar in all other respects to testcross progeny. F4 plants derived by self pollination of homozygous *rmr1-1* F3 plants were also normal in appearance and similar in all respects to the previous F3 plants. A single F3 plant homozygous for *rmr2-1* was crossed to a *rmr2-1* heterozygote from a separate lineage to generate a family where *rmr2-1* homozygotes and heterozygotes could be compared. Aside from differences in anther pigmentation, *rmr2-1* homozygotes were indistinguishable from their heterozygous siblings. Although it remains possible that prolonged exposure of the genome to defects in RM*R1* and RMR2 functions could have

pleiotropic consequences, we currently have no indications that they are required for proper plant development, at least in the A632, A619, or W22 backgrounds.

[00281] **rmr1 and rmr2 Mutations Prevent Mu Element Methylation.** Given the observation that *Mop1* affects both paramutation and Mu methylation (Example 2), we were curious to see if the rmr mutations did the same. Because the background in which the rmr mutations was isolated lacked full length *MuDR* elements (data not shown), it was possible to directly observe the effect of these mutations on endogenous *Mu1* elements in the absence of the transposase. DNA from three families that were segregating 1:1 for homozygous mutant and heterozygous siblings was analyzed for the methylation status of *Mu1* sequences (see Example 2 for details). Four of four *rmr1-1/rmr1-1* plants had unmethylated *Mu1* sequences while four of seven *rmr1-1/+* plants had clearly methylated *Mu1* sequences (Figure 32A). The other three *rmr1-1/+* plants did not have methylated *Mu1* sequences (Figure 32A). The correlation between *Mu1* methylation and *rmr2-1* genotypes was perfect; eight of eight *rmr2-1/rmr2-1* plants had no detectable *Mu1* methylation and 10/10 *rmr2-1/+* plants had heavily methylated *Mu1* sequences (Figure 32B). These results strongly suggest that Mu element hypomethylation is a generic effect of mutations affecting paramutation rather than a specific effect of *Mop1*.

[00282] **Summary.** Using a seedling-based genetic screen, we identified three maize loci (*Mop1*, rmr*1* and rmr2) whose trans-acting functions are required to maintain gene silencing that occurs as the result of paramutation at the *pl1* locus. In all plants homozygous for mutations in *Mop1*, rmr*1*, or rmr2, *Pl'* is expressed at a high level indistinguishable from *Pl-Rh* (Example 1). While the reduced expression state (*Pl'*) is most frequently restored upon outcrossing to non-mutant plants, an increased expression state indistinguishable from *Pl-Rh* can be meiotically heritable. Because *pl1* paramutation is associated with changes in *pl1* transcription, our working model is that RM*R1*, RMR2, and *MOP1* functions are involved with the maintenance of specific chromatin structures that prohibit high levels of *pl1* transcription.

[00283] Additional rmr loci are clearly involved in *Pl'* repression. Our reported genetic screen represents only 10% theoretical saturation. This estimate is based on the apparent mutation rate of one detectable hit per gene per 1000 genomes screened and the roughly 95% probability that 5000 screened genomes would yield at least one detectable mutation in every given gene. These numbers suggest that six to ten such rmr-like loci exist in maize. Indeed, five additional rmr loci have already been identified in ongoing genetic screens (see Example 6, J. Hollick, unpublished).

[00284] Paramutation silencing at the *pl1* locus not only leads to repression of *Pl-Rh* expression, it also affects the way in which the *Pl-Rh* allele is regulated (Hollick et al. 2000). *Pl-Rh* expression is light-insensitive while *Pl'* expression is light-dependent. As most *pl1* alleles are regulated by light cues, the observations that rm*r1* and rmr2 mutations have no effect on pl-A632 or pl-W22 suggests that RMR functions do not mediate light-induction. Rather, we hypothesize that RMR functions are used in maintaining a specific regulatory state unique to *Pl'*. The *Pl-Rh* state then must actively prevent or avoid RMR action. Two general and non-exclusive modes of action are considered: 1) rmr RNA or proteins are components of a heritable chromatin structure that affects *pl1* transcription, 2) RMR functions facilitate alterations or maintenance of a heritable chromatin structure. Previous work has shown that paramutant *pl1* and *r1* alleles require continued allelic interactions with another paramutant or paramutagenic partner (Styles and Brink 1968; Hollick and Chandler 1998). It is possible that RMR functions actually mediate these allelic interactions through homology searching or specifying intranuclear positions.

[00285] It is not known whether the rmr mutations described represent complete loss-of-function alleles. In fact, the observation that *pl1* RNA levels are significantly lower in homozygous *rmr1-2* plants versus *rmr1-1* plants hints that the two alleles have different levels of activity. Nonetheless, mutations at both the rm*r1* and rmr2 loci are completely recessive suggesting that neither RM*R1* or RMR2 functions are dosage sensitive as are many of the genes required to mediate examples of position effect variegation seen in Drosophila (reviewed in Weiler and Wakimoto 1995).

[00286] Despite similar effects on *pl1* RNA levels, the frequency of meiotically-heritable *Pl'* to *Pl-Rh* changes is clearly different in the rm*r1* mutants versus *rmr2-1* mutants. Even in *rmr1-2* homozygotes where the relative level of *pl1* RNA is lower than in *rmr2-1* homozygotes, *Pl'* changes to a *Pl-Rh* state more frequently in homozygous *rmr1-2* plants. This set of results implies that derepression of *Pl'* in somatic tissues is, by itself, insufficient to allow a meiotically-heritable change of *Pl'* to *Pl-Rh*. In addition to *Pl'* heritably changing to *Pl-Rh* more frequently in rm*r1* mutant plants, those alleles that remain *Pl'* upon transmission appear to be less paramutagenic than those transmitted from *rmr2-1* mutant plants. One interpretation is that RM*R1* and RMR2 functions are distinct in terms of their role(s) in maintaining meiotically-heritable repression of *Pl'*. This idea is supported by the finding that *rmr1-1* leads to loss of a post-transcriptional regulatory step while *rmr2-1* leads to loss of a transcriptional-based mechanism.

[00287] Although *Pl'* heritably changes to *Pl-Rh* at different frequencies between individual *rmr1-1/rmr1-1* plants, similar frequencies were observed independent of female

versus male transmission in several reciprocal crosses (Table 10). In these families, the frequency of meiotically heritable changes of *Pl'* to *Pl-Rh* was established early in development prior to the point that cell lineages diverged to specify the lateral versus apical infloresence meristems. Frequencies of heritable *Pl'* to *Pl-Rh* changes were distinct, however, between each set of reciprocal crosses. This observation implies that different frequencies can be established and maintained through most of sporophyte development with relatively high fidelity. It remains unclear as to whether *Pl'* actually changes to a meiotically-heritable *Pl-Rh* state during early development or whether the probability of such changes later in development are preset early on.

[00288] RM*R1* and RMR2 functions do not appear to be involved in general gene control mechanisms required for development. To date, plants homozygous for the *rmr1-1*, *rmr1-2* or *rmr2-1* alleles have been morphologically and developmentally indistinguishable from their heterozygous siblings. This absence of morphological defects, even after three generations of selfing, suggests that rm*r1* and rmr2 are unlikely to be maize orthologues of the Arabidopsis ddm1 or met1 genes. Mutations of ddm1, a SWI2/SNF-2-like gene (Jeddeloh et al. 1999), and dominant-inhibitors of met1, a DNA methyl transferase enzyme, have broad and cumulative effects on Arabidopsis development (Kakutani et al. 1996; Ronemus et al. 1996; Finnegan et al. 1996). Because mutations in *Mop1* also appear to have effects on plant development (Example 1), it appears that the rm*r1* and rmr2 loci define a class of genetic functions distinct from *MOP1*. Alternatively, RM*R1* and RMR2 may participate in developmental pathways similar to *MOP1* but have redundant developmental functions. Double mutant combinations are currently being synthesized to test this possibility.

[00289] Several other genes required to maintain transgene silencing in Arabidopsis have been recently described. Some of these genes like sgs1, sgs2, sgs3 (suppressor of gene silencing) and sde1, sde2, sde3, and sde4 (silencing defective) are required for post-transcriptional silencing (Elmayan et al. 1998; Mourrain et al. 2000; Dalmay et al. 2000) while others, hog1 (homology-dependent gene silencing 1), sil1, sil2 (silencing), and mom1 (Morpheus' molecule 1) are required for transcriptional-based silencing (Furner et al. 1998; Amedeo et al. 2000). Given that paramutation at the *b1* and *pl1* loci affect heritable states of transcriptional control (Patterson et al 1993; Hollick et al. 2000), the rmr genes could potentially be related to this latter class of Arabidopsis genes. Paramutation has not been described in Arabidopsis.

EXAMPLE 4:

Isolation of a Dominant Mutation, *Mop2-1*, and two recessive mutations, *mop3-1* and *CC2343*, using the *B'* screen

1. Materials and Methods.

[00290] **Plant Stocks**. All plant stocks contained dominant functional alleles for all the genes encoding the anthocyanin biosynthetic enzymes required in vegetative plant tissues. Because transcription of these genes in vegetative plant tissues is controlled by *pl1* in combination with *b1* or *r1*, the specific *b1*, *pl1* and *r1* alleles are indicated for relevant stocks. One exception is the distinction between *Pl'*-mahogany (*Pl'*) and *Pl-Rh*oades (*Pl-Rh*) (Hollick et al., 1995). Many stocks possess the *R-g* allele of *r1* (no expression in the seed or plant), which precludes reliable scoring of *Pl'* versus *Pl-Rh*. In these stocks, we have used *Pl*to indicate the presence of either *Pl-Rh*, or its spontaneous derivative *Pl'*.

[00291] A *gl2* b wt, Pl, *R-g* (inbred K55 background) stock, as well as *B-I PlR-g* (inbred W23 background) and *B' PlR-g* (inbred K55 background) stock were originally obtained from E.H. Coe, Jr. (University of Missouri, Columbia). These stocks were used by V.L. Chandler to generate *gl2 B'* wt and *gl2 B-I* wt stocks. To test whether *Mop2-1* affects *pl1* paramutation, our *Mop2-1* stocks were crossed with stocks containing the *R-r* allele to facilitate reliable scoring of the *pl1* genotype. These included a *gl2* b wt, *Pl-Rh*, *R-r* stock generated by V.L. Chandler, and a y1 *Pl'* sm, PRR stock originally obtained from the Maize Cooperation Stock center.

[00292] **Genetic screen**. A screening population was generated by treating *B'*, *Pl-Rh*, *R-g* (inbred K55 background) pollen with ethyl methanesulfonate (EMS; 0.063% as described by Neuffer and Coe, 1978), and using treated pollen to pollinate *gl2 B-I* wt, *Pl-Rh*, *R-g* (stock generated by V.L. Chandler--mix of W23/K55 inbred stocks) ears, producing M1 seed. The *gl2* and wt loci flank the *b1* gene. In wild-type stocks, *B'* will paramutate *B-I*, and all progeny will be light, whereas rare plants having *B-I* pigmentation levels may indicate the presence of a mutation preventing the establishment of paramutation. The M1 seed was planted to screen mature plants for rare individuals having *B-I* pigmentation levels. Presence of the recessive *gl2* and wt markers in the *B-I* ear parent enable rapid identification of any self-pollination contaminant offspring. This is important because self-contaminants would yield the desired rare phenotype of *B-I* pigment levels.

[00293] Over 7300 M1 seed was planted in the summer of 1993. One exceptional darkly-pigmented individual was identified (KK1238-1). This individual was self-pollinated, and

outcrossed to several tester stocks (*gl2 B-I* wt, *gl2 B'* wt, and *gl2* b wt). The results of these and subsequent crosses (detailed below) demonstrated that KK1238-1 carried a heritable dominant mutation capable of inhibiting *B-I* paramutation. This dominant mutation has been designated *Mop2-1*.

[00294] Many of the M1 plants (other than KK1238-1) that showed a *B'* phenotype were self-pollinated, and M2 families were screened in sand benches for the presence of rare darkly-pigmented seedlings resembling *B-I*-like plants among siblings that were essentially green. One family (KK1191-1X) segregated 1/4 darkly-pigmented plants suggesting the presence of a recessive mutation. Dark and light individuals along with appropriate testers were transplanted. Another ~100 families were screened, but no other mutations were found. Crosses were performed with the new putative mutant to test for heritability, bulk up seed and begin complementation tests with other mutants such as those described in Example 1 and Example 3.

[00295] **Molecular Markers** Map position of the *Mop2-1* mutation was confirmed using simple sequence repeat (SSR) molecular markers. A large number of these PCR-based markers are available for maize (http://www.agron.missouri.edu/ssr.html). The SSR markers that are tightly linked to *Mop2-1* and were used to follow *Mop2-1* segregation are bnlg1017, bnlg1338 and umc1823.

2. Identification and Characterization of a Dominant Mutation, *Mop2-1*

[00296] The facts that *B'* is extremely stable and that paramutation always occurs when *B'* and *B-I* are brought together in a heterozygous individual enable a simple genetic screen to identify mutations involved in paramutation. *B'* pollen was treated with the mutagen ethyl methanesulfonate (EMS), and used to pollinate *gl2 B-I* wt ears. The *gl2* wt recessive marker loci, which flank the *b1* gene, enable the identification and elimination of any *gl2 B-I* wt pollen self-contaminants, and can also be used to follow chromosome 2 segregation in subsequent generations. Over 7300 M1 seed were grown to maturity and screened for dark plants that may carry a dominant mutation that disrupts the establishment of paramutation. One exceptional dark plant was recovered.

[00297] A series of crosses were done to confirm the presence of a heritable mutation and further characterize its behavior. This exceptional dark plant was crossed to a *gl2 B'* wt tester plant, and all 15 *B'* progeny were subsequently crossed to *gl2 B-I* wt to determine which individuals carried the dominant mutation capable of inhibiting paramutation (Figure 23). To determine whether this dominant mutation identifies an independent locus involved in

paramutation, or a mutation in the *B'* allele which abolishes its paramutagenic properties, we tested for co-segregation of the mutation with the *B'* allele. The *B'* allele originating from the EMS treated pollen was flanked by wild-type alleles of the *gl2* and wt loci (*Gl2* and Wt), whereas the *B-I* allele carried the recessive *gl2* wt markers. These markers were used to separate progeny of the above cross with *gl2 B'* wt into those inheriting the *Gl2 B'* Wt chromosome, and those inheriting the *gl2 B-I* wt chromosome (Figure 23). One individual was recombinant in the *gl2* wt interval and was therefore not informative with respect to which *b1* allele had been inherited, though the presence of dark progeny in a testcross with *gl2 B-I* wt demonstrates that it inherited the mutation. Of six individuals inheriting the *Gl2 B'* Wt chromosome, all six gave rise to dark progeny when crossed to *gl2 B-I* wt consistent with each of them carrying the dominant mutation. If the mutation were unlinked to *b1*, we would expect that only half of these individuals would inherit the dominant mutation and give dark progeny in the next generation. The likelihood that all six individuals would inherit an unlinked mutation by chance is small ($p = 0.016$), therefore it is most likely that the mutation is linked to *b1*.

[00298] To further determine whether this linked mutation is disrupted in the *B'* allele or in a linked but independent locus, the progeny of these six individuals were further examined. Given the fact that a double recombination event separating the *B'* allele from its flanking *Gl2* and Wt markers would be quite rare, if the mutation is at *B'* we would expect that all *Gl2* Wt progeny would be dark and all *gl2* wt progeny should be light. This expectation was not met. Eleven percent of the *gl2* wt progeny were dark, and 12.5 % of the *Gl2* Wt progeny were light. These results are most consistent with the mutation residing outside the *gl2* wt interval and thus independent of the *b1* locus. The fact that *Gl2* wt recombinant progeny inherited the mutation (dark plants) and *gl2* Wt recombinant progeny did not (light plants) demonstrates that the mutation lies distal to the *gl2* locus.

[00299] As additional confirmation of this conclusion, nine progeny of the same original cross to *gl2 B'* wt (Figure 23) inherited the *gl2 B-I* wt chromosome, though as described above, all nine were light (*B'* phenotype) confirming that the *B-I* allele was still paramutable. These nine individuals were testcrossed to additional *gl2 B-I* wt tester stocks to determine whether they carried the dominant mutation. Consistent with the mutation residing on the homologous chromosome, seven of these individuals did not carry the mutation and gave all light progeny, whereas two individuals gave dark progeny indicating they had inherited the linked mutation, but that it must reside outside the *gl2* wt interval.

[00300] Thus, this mutation identifies a locus, distinct from *b1*, which appears to play a role in paramutation. Based upon subsequent analyses described below which reveal

similarities with the recessive mediator of paramutation 1 mutations, we have designated this locus mediator of paramutation 2, and the mutation *Mop2-1*.

3. Penetrance of *Mop2-1* vs. spontaneous paramutation of *B-I*.

[00301] Though segregation of the mutation could be followed by the presence of dark progeny, the frequency of dark progeny in some of the above crosses was less than predicted. In crosses of *B'/B' Mop2-1*/mop2 x *B-I/B-I* mop2/mop2, 50% of progeny should be dark because they inherit the *Mop2-1* mutation, yet in some families the frequency of dark progeny was much lower (30-35%). One possible interpretation is that *Mop2-1* is not fully penetrant in preventing paramutation. Another possibility is that the *B-I* allele of the tester plants is spontaneously paramutating to *B'* on a stochastic basis. Spontaneous changes of *B-I* to *B'* occur regularly in both standard and *Mop2-1* stocks, and could easily account for the discrepancy (~35% observed vs. 50% expected) observed in some families. Plants in which this occurs would be light even though they carry *Mop2-1*. Consistent with this interpretation is the fact that spontaneous paramutation of *B-I* to *B'* occurs in wild-type stocks, and often occurs more frequently in some families than others. The distribution of color scores among the progeny of 9 individuals that carried the *Mop2-1* mutation are shown in Figure 26. Color scores were used as many individuals did not fall into clear categories of light or dark, but rather had intermediate phenotypes. Color scores of 1-3 are typical of a *B'* individual, and 6-7 are typical of a *B-I* individual. Both sheaths and tassels were scored. Some families gave a clearly bimodal distribution of color scores with approximately 1:1 *B-I* to *B'* progeny (e.g. Families 4, 5, 7). Other families gave considerably more intermediate individuals (color scores of 4-5, e.g. Families 2, 6, 8).

[00302] The fact that *B' Mop2-1*/mop2 x *B-I* individuals inheriting *Mop2-1* can be light is also apparent in the fact that two light progeny of the original dark plant inherited recombinant *gl2 B-I* wt chromosomes carrying *Mop2-1*. These progeny were light upon outcross to *gl2 B'* wt, consistent with either incomplete penetrance of the mutation or spontaneous paramutation of the *B-I* allele, but the presence of *Mop2-1* was established upon outcrossing each individual to *gl2 B-I* wt and observing dark progeny.

[00303] ***Mop2-1* also prevents the establishment of *pl1* paramutation** To determine whether mop2 can affect additional paramutable loci in trans or is restricted to the linked *b1* locus, we tested whether it could inhibit the establishment of *pl1* paramutation. We crossed an individual heterozygous for the *Mop2-1* mutation with a stock homozygous for the *Pl-Rh* and *R-r* alleles. All progeny from this cross had fully-pigmented anthers (*Pl-Rh*) which enabled us to confirm that the *Pl-Rh* allele in the *Mop2-1* plant had not spontaneously

changed to *Pl'*. Progeny of this cross, half of which should carry the *Mop2-1* mutation, were crossed with a paramutagenic *Pl'* stock. Two of these individuals gave progeny segregating 1:1 for dark (*Pl-Rh*) and light (*Pl'*) anthered plants suggesting they carried the *Mop2-1* mutation, whereas a third individual gave all *Pl'* progeny consistent with the parent individual lacking the *Mop2-1* mutation. The presence of dark-anthered progeny demonstrates that the *Mop2-1* mutation is able to inhibit the establishment of *pl1* paramutation. The 1:1 segregation ratio (total for both families 17:18) observed among the progeny further suggests that the *Mop2-1* mutation is fully penetrant with respect to the inhibition of *pl1* paramutation. This observation, together with the fact that some crosses with *B-I* yield 1:1 segregation ratios, suggests that the *Mop2-1* mutation is fully penetrant and that instances in which less than 50% darks are observed are more easily explained by spontaneous paramutation of *B-I* to *B'*. These results are also consistent with observations that *B-I* is less stable than *Pl-Rh*, i.e. more likely to undergo spontaneous paramutation.

[00304] **Phenotype of homozygous *Mop2-1*.** To determine whether this mutation represents a true dominant or a semi-dominant allele, we have generated homozygous mutant individuals. Though the original exceptional dark individual in the M1 screening population had been self-pollinated in an effort to generate individuals homozygous for the mutation, nearly all progeny of this self-pollination were unhealthy or failed to thrive such that testcrosses for the identification of homozygous individuals were difficult to obtain. This is presumably due to the presence of additional mutations resulting from the EMS mutagenesis that become homozygous in the M2 progeny. Instead, we took advantage of the fact that *Mop2-1* heterozygous individuals resulting from the *pl1* experiments described above were considerably more vigorous than previously identified *Mop2-1* stocks, presumably due to hybrid vigor from multiple outcrosses to distinct genetic stocks. Several of the heterozygous *Mop2-1* individuals identified by the presence of dark red anthers were self-pollinated. Progeny (*B'*) were self-pollinated and testcrossed to *B-I* to determine their mop2 genotype. All 13 testcross progeny of one individual possessed the *B-I* phenotype, indicating that the parent had been homozygous for the *Mop2-1* mutation. Progeny from the self-pollination of this individual were grown in our winter nursery in Hawaii. Progeny from the self-pollinations of two sibling individuals shown to be heterozygous for the *Mop2-1* mutation were also grown as a control.

[00305] Plants of these self-pollinations were initially scored about 2 weeks prior to anthesis. No obvious phenotypic differences were noted at that time, though the progeny of the homozygous individual were somewhat less vigorous than most progeny of the heterozygous individuals. As the progeny of the homozygous *Mop2-1* individual approached anthesis, they darkened. Progeny from the self-pollinations of two heterozygous *Mop2-1*

individuals showed two phenotypic classes: several individuals were lightly pigmented and quite vigorous, some of which were already flowering; whereas a few individuals were spindly, much later flowering, and somewhat darker than their sibs. Furthermore, some additional individuals were less than half the height of their sibs, quite spindly, and showed no hope of producing an ear. Among the slightly darker progeny of the heterozygous individuals, and the progeny of the homozygous individual, were some individuals showing a tassel-seed phenotype. Over the next week or two, some of these individuals continued to darken such that some approached a *B-I*-like phenotype (Figure 25). Analysis of a linked simple sequence repeat (SSR) locus, and testcrosses for a majority of individuals in these families confirmed that the darker individuals were homozygous for the *Mop2-1* mutation whereas the lighter sibs were heterozygous or homozygous for the wild-type allele. This difference in pigmentation correlates with increased *b1* transcript levels in the homozygous *Mop2-1* individuals relative to their heterozygous siblings (Figure 26A).

[00306] To determine whether the darker phenotype of the homozygous *Mop2-1* individuals would result in a heritable alteration of the *B'* allele, some of the darker homozygous individuals were also crossed to a B-Peru tester. B-Peru gives purple kernel color but extremely weak plant color such that *B'* or *B-I* plant pigment is readily apparent. All progeny of this cross had typical *B'* pigment levels, suggesting that the effect of homozygous *Mop2-1* had not produced a heritable alteration of *B'*.

[00307] ***Mop2-1* Summary.** *Mop2-1* represents a novel dominant mutation able to inhibit the establishment of paramutation at *b1* and *pl1*. In this sense, *Mop2-1* is distinct from *Mop1*, mop3, rm*r1* and rmr2. Plants homozygous for this mutation also show recessive phenotypes such as increased pigment and *b1* transcript levels, and developmental abnormalities. These recessive pigment phenotypes are similar to those observed for mutations in *Mop1*, mop3, rm*r1* and rmr2, and the developmental abnormalities similar to mutations in *Mop1*, mop3 and *CC2343*.

4. Isolation and Characterization of recessive mutations, *mop3-1* and *CC2343*

[00308] Two new recessive mutants were isolated in seedling and mature plant screens using *B'*. Examples of the phenotypes are shown in Figure 27. Both of these mutations appear to represent unique genes, which increase the RNA levels of *B'* and *mop3-1* decreases the methylation of *Mu1* elements. These results indicate that the two genes identified by these mutations are required for maintenance of paramutation at *B'* and *mop3-1* and for maintenance of the hypermethylation of *Mu1* elements.

[00309] **Isolation of *mop3-1*** All but one of the M1 plants from the screen described for the isolation of *Mop2-1* had a wild-type *B'* phenotype, yet they could carry new recessive mutations. These M1 plants (other than the plant carrying the *Mop2-1* mutation) were self-pollinated to generate M2 families. M2 families were screened in sand benches for the presence of rare darkly-pigmented seedlings resembling *B-I*-like plants among siblings that were essentially green. One family (KK1191-1X) segregated 1/4 darkly pigmented plants (example of phenotype in Figure 27A) suggesting the presence of a recessive mutation.

[00310] The KK1191-1 family showed good penetrance of the mutant phenotype, and additional genetic tests of this mutation were performed. To bulk up the family, a plant homozygous for this mutant and homozygous for *B'* was crossed to a plant that was homozygous for *B-I*. Ninety of the resulting seed were planted. Approximately 75 plants germinated; all of them had the *B'* phenotype, consistent with the new mutation being recessive and that the *B'* state was not heritably changed to *B-I* in the homozygous mutant. Several of these plants were crossed to siblings to test for heritability. In one family of 15 plants, four showed the mutant phenotype, consistent with the segregation of a single recessive mutation.

[00311] **Isolation of *CC2343*.** The *CC2343* mutation was isolated using a different screen as compared to that which led to the identification of the other mutations. Pollen from *B'* plants was treated with EMS and used to pollinate *B'* ears. A total of 1464 M1 seed were planted and screened for dark plants, which could represent a dominant mutation that relieved the silencing associated with the paramutant *B'* state. One dark plant was observed and designated *CC2343*. The mutant plant made no fertile ear, so it could only be crossed as male onto testers. The only cross that produced seed was to DS1630 (b *R-g* PI). Thirty-four seed were obtained and planted in the spring of 2000, only 22 germinated. One of the 22 plants was dark at its base with a *B'* phenotype in its tassel (N182-16), while the rest of the plants had the *B'* phenotype. If the original dark plant had carried a dominant mutation that increased the expression of *B'*, the expectation is that ½ the plants should have been dark. This expectation was clearly not met. Three possibilities were considered: first, the original plant did not represent a new mutation; second there was a low penetrance due to background or environmental effects; third, the original dark plant contained two unlinked mutations and both needed to be present as heterozygotes for the phenotype to be observed. A prediction of the third hypothesis is that ¼ of the plants should have been dark because they received both unlinked mutations. This was not observed. However, approximately one-third of the seeds did not germinate (12/34), which could skew the segregation ratios. Further experiments described below demonstrate that at least one new recessive mutation is segregating in these plants.

[00312] To determine if recessive mutations were present in the plants, each plant was self-pollinated and progeny planted and scored for light versus dark phenotypes. Six self-pollinated plants yielded seed, which were planted in Fall 2000. If half of the original N182 plants had carried a recessive mutation, then the expectation is that half of these six families (N268, N271, N273, N275, N276, O136) would segregate dark plants in the next generation. One family (N268) segregated only green and light (*B'*) plants. Five of the six familes (N271, N273, N275, N276, O136) each produced dark (9), medium dark (9) and lightly pigmented (16) plants, as well as green plants (17; b/b genotype). Figure 27C,D illustrates these phenotypes. The medium dark plants were healthy, but the very dark plants were most often short and sickly. Only one made an ear, and most had small tassels that shed poorly. The frequency of N182 plants that gave rise to mutant progeny is more consistent with the hypothesis that two independent mutations were segregating (expected frequency 0.75), rather than the hypothesis of a single mutation present in the original isolate (0.5). However, the fact that only 6/22 plants could be tested precludes a definitive conclusion.

[00313] Subsequent crosses have confirmed the presence of at least one mutation that increases *B'* pigment, but the poor germination and health of these plants has made it impossible to definitively conclude whether one or two mutations are segregating in these lines based simply on segregation ratios. The medium dark plants were self-pollinated, and the medium dark and dark plants were crossed to their *B'* siblings and outcrossed to *B'* testers. If the dark and medium dark plants were homozygous for a recessive mutation, then the outcrosses to *B'* testers should produce progeny uniformly *CC2343/+ B'/-*. Four families that resulted from dark plants crossed to *B'* were planted and scored; all forty-two had the *B'* phenotype, consistent with a recessive mutation, not a semi-dominant mutation, in the dark plants. In the next season both the medium dark (dark sheaths and husks, but lighter, more *B'*-like tassel pigment; Figure 27D) and dark plants (dark pigment in sheaths, husks and tassel; Figure 27C) were crossed with *B' CC2343/+*. When possible they were also self-pollinated. In Fall 2001, the number of dark, medium dark and light plants were scored. Unfortunately, germination was very poor, such that statistically significant numbers of plants were not available for scoring in most families. Some families resulting from self-pollination of medium dark and dark plants produced all medium dark or dark plants, consistent with both phenotypic classes being homozygous for a mutation. However, some families resulting from dark or medium dark crosses with *CC2343/+* produced only light plants. This could mean that there are indeed two different recessive mutations segregating in these lines, and additional experiments are underway to further explore this idea.

[00314] **Complementation Tests for *mop3-1* and *CC2343*.** Complementation tests are in progress between *mop3-1* and *CC2343* and all of the other mutants isolated to date

(Examples 1, 3, 6). The data from the initial set of experiments is summarized in Table 11. All of these crosses were between homozygous *mop3-1 B'* plants and heterozyotes for each of the other mutations. Thus, if *mop3-1* is a mutation in the same gene as the mutation being tested, the expectation is 50% dark plants and 50% light plants when scoring for effects on *B'*, and 50% ACS scores 1-4 and 50% ACS scores 5-7 when scoring for effects on *Pl'*. The *mop3-1* mutation clearly defines a distinct gene from *Mop1*, rmr2, rmr8 and rmr9 as all the progeny are *B'* with low ACS. Crosses of *mop3-1* with *rmr11-1* mutants showed dark plants; at least 1 dark plant was observed in ¾ of the families, but the numbers were significantly lower than that expected for noncomplementation. We hypothesize that *mop3-1* can interact with *rmr11-1*, as has been observed with other mutants (see Example 6). Crosses between *rmr1-1* and *mop3-1* produced a few dark anthered plants, but no increase in *B'* pigment. The low number of these individuals is not consistent with noncomplementation.

Table 11. Complementation Data for *mop3-1* and *CC2343*

mop3-1	Plant Phenotype		# Ears Scored	Anther Color Scores*						
	B'	Dark		1	2	3	4	5	6	7
rmr1-1	23		5	2	6	4	3	1	1	2
Mop1-1	48		3	Y						
rmr2-1	27		3	9	10	5	1	1		
rmr9-1	16		2	6	3	3	1			
rmr11-1	41	8	4	20	13	1		1	2	2
rmr8-1	46		4	4	20	9	3	1		

CC2343	Plant Phenotype		# Ears Scored	Anther Color Scores*						
	B'	Dark		1	2	3	4	5	6	7
rmr1-1	71		4	4	15	12	10	11	9	2
Mop1-1	76	35	6	Y						
rmr2-1	24		1	7	13	4				
rmr11-1	9	1	2	3	1	1		1		

[00315] *Not all plants could be scored for anther color because in almost all families some of the tassels were destroyed prior to scoring. Therefore, more plants were scored for plant body phenotype than for ACS score.

[00316] Families noted as Y in the anther color score 1 column were *R-g* (yellow) and therefore it was not possible to score anther color.

[00317] The complementation tests with the *CC2343* mutation suggest that *CC2343* is in a gene distinct from rmr2. The experiments with the other mutations are less clear as the segregation ratios do not fit either the complementation or noncomplementation hypotheses. The simplest explanation is that *CC2343* may interact with *Mop1-1*, *rmr1-1* and *rmr11-1*, but futher experiments will be necessary to test this hypothesis.

[00318] ***Mu1* Methylation Tests with *mop3-1*.** Sibling plants segregating *mop3-1* were analyzed for the methylation status of *Mu1* elements, as described in detail for *mop1-1* in Example 2. DNA was extracted from light (heterozygous or homozygous wild type) and dark (homozygous for the mutation) plants, digested with *Hin*fI and probed with *Mu1*. As in the case with *mop1-1* (Example 2), *Mu1* elements in the mutant plants were hypomethylated relative to *Mu1* elements in their wild type siblings (equivalent data was obtained as that shown in Figure 15 for *mop1-1*).

[00319] The *mop3-1* and *CC2343* mutations share several features with *Mop1-1*. Similar to *Mop1-1*, the dark pigment phenotype of homozygous *mop3-1* and *CC2343* individuals is the result of increased *b1* transcript levels (Figure 26B). Also similar to *Mop1-1*, *mop3-1* and *CC2343* occasionally show developmental effects when homozygous. Families generated from the self-fertilization of *mop3-1* or *CC2343* homozygotes were screened for the appearance of new phenotypes. Many of the plants showed severe developmental aberrations, including small or missing ears, runty tassels and often strongly feminized tassel structures (Figure 27E,F).

[00320] The plants were also very small. With *mop3-1*, these phenotypes occurred in essentially every plant that has been homozygous for multiple generations, but were less frequent in plants heterozygous for the mutation in the previous generation. Although fewer generations have been examined with *mop3-1* and *CC2343* relative to *Mop1-1*, the developmental defects in *mop3-1* and *CC2343* appear more frequently and are more severe.

[00321] **Summary of *mop3-1* and *CC2343* results.** The complementation tests completed to date suggest that the *mop3-1* and *CC2343* mutations represent unique genes. Both *mop3-1* and *CC2343* are required to maintain the reduced expression state associated with B' paramutation. Similar to mop1 mutations, *mop3-1* and *CC2343* do not heritably alter the reduced expression state as B' segregates in subsequent generations in the absence of the mutation. Also similar to *mop1-1*, homozygous *mop3-1* and *CC2343* individuals show increased *b1* transcript levels and developmental abnormalities. The *mop3-1* mutation is also required to retain the extensive methylation associated with silenced Mutator elements. Experiments to examine the effects of *CC2343* on *Mu1* methylation are in progress.

Example 5

Four Mutations Defective in Paramutation Activate Previously Silent Transgenes

[00322] **Overview.** Genetic studies in the 1950's revealed several examples of gene regulation that are variable, unstable, but heritable. These phenomena included paramutation and the cycling of transposable element activity in maize. Numerous models were discussed, with a common theme that global chromosomal levels of control were operating (reviewed in Chandler and Vaucheret 2001). During the past twelve years extensive studies on transgene expression in plants has revealed a wide range of gene silencing phenomena (reviewed in Fagard and Vaucheret 2000). Transgene silencing can occur either at the transcriptional (TGS) or post-transcriptional level (PTGS). It can happen in cis, affecting single transgene copies or in trans, affecting unlinked sequences (including transgenes, endogenous genes or viruses) that share high sequence similarity. TGS is often associated with sequence homology and DNA methylation in the promoter regions. PTGS correlates with homology in the transcribed regions and DNA methylation in the transcribed region and the accumulation of small RNAs (21-25 nt). It is not yet clear whether these correlations are causes or consequences of gene silencing. Paramutation at *b1* is more similar to TGS rather than PTGS, because the transcription rate of paramutated alleles is decreased (Patterson et al. 1993).

[00323] Given that paramutation shares some features with transgene silencing (reviewed in Chandler et al. 2000), we tested whether mutants defective in paramutation might have effects on transgene silencing. Previous work done in the Chandler lab had generated several transgenic lines, all of which contained various B transgenes that had been produced to study anthocyanin gene regulation. The B gene encodes a transcription factor that activates the biosynthetic pathway. Expression of the B gene in a particular tissue will give rise to purple pigment in that tissue. All the transgenic lines were generated by particle bombardment of immature maize embryos (C. Carey, D. Selinger and V. Chandler, unpublished data; see Selinger et al. 1999 for detailed methods). Transgenic lines that contained two different B constructs were used. In both cases the transgene was silent in vegetative and reproductive plant tissues yet it remained active in the aleurone layer of the seed. Each independent transgene segregated as a single genetic locus, but DNA blot analyses revealed multiple copies of the construct had inserted into the genome. Figure 28 diagrams the constructs that were used to generate each of the transgenic lines. Transgenic line VLC44-27A carries a 35S promoter driving a *B-I* genomic construct. Expression of this transgene had never been phenotypically detectable in the plant. The BBBS transgene (VLC40-64A) carries the promoter from B-Bolivia fused to the *B-I* genomic region (Figure 28;

Selinger and Chandler, 2001). The original transgenic line was expressed in both the plant (leaf blade primarily) and in the aleurone layer of the seed. However, the plant pigmentation was lost in subsequent generations.

[00324] **Four mutants with defects in paramutation relieve transgene silencing.** For these experiments we combined each of four different mutations (*Mop1-1*, *Mop2-1*, *rmr1-1* and *rmr2-1*) with lines containing the silent transgenes. The resulting F1 plants containing the transgene (hemizygous) were identified via PCR and DNA blots. All of these individuals were also heterozygous for a particular mutation. No activation of the transgenes was observed in this generation. These were backcrossed to plants homozygous for the particular mutation. This protocol yielded families that were segregating for the transgene and heterozygous or homozygous for the mutation being tested. For the experiments with *Mop1-1*, *rmr1-1* and *Mop2-1*, seeds carrying the transgene could be identified by the transgene's expression in the aleurone layer, which resulted in medium purple kernels. [It is not known why both transgenic lines were completely silent in vegetative and floral tissues, but expression was observed in the differentiated aleurone layer.] The medium purple kernels were planted and the plant and anther pigment phenotype was determined. If each of the mutations activated the transgene when homozygous, we expected ratios of 1:1 for active:inactive (presumably homozygous and heterozygous for the mutation, respectively). This expectation was met for both *Mop1-1* and *rmr1-1* with the two different transgenes (Table 12). Examples of the phenotype of each of the mutants and heterozygous siblings with the transgene, 35S*B-I*gen (VLC44-27A), are shown in Figure 29A-D. Examples of activation of the BBBS transgene by *rmr2-1* are shown in Figure 29E.

Table 12: Activation of silent transgenes

	35S*B-I*genomic			BBBS		
	No. Active	No. Silent	X^2	No. Active	No. Silent	X^2
Mop1-1 (1:1)	213	192	1.1	27	26	0.02
rmr1-1 (1:1)	55	52*	0.09	15	13	0.14
Mop2-1 (1:1)	16	32	5.3**	N.D.	N.D.	
rmr2-1 (1:3)	26	95	0.79	28	96	0.39

[00325] Forty-four of these individuals had ACS scores between 1-4, consistent with a genotype of *Rmr1/rmr1-1*. Eight individuals had ACS scores of 6-7. This could be due to reversion of *Pl'* to *Pl-Rh*, with these individuals being *Rmr1/rmr1-1* and not activating the transgene. Alternatively, the plants with ACS6-7 could have been *rmr1-1* homozygotes, but the transgene was not activated. Unfortunately testcrosses of these individuals were not obtained so the *rmr1* genotype could not be assessed. However, the observed segregation ratios of plants with active and inactive transgenes (in the two families where this was observed) are most consistent with the first hypothesis.**P is < 0.05, significant deviation from 1:1 hypothesis; N.D. = not determined

[00326] When active, neither of the transgenes used were ever expressed in the anthers. This enabled us to use anther color to follow the presence of heterozygous and homozygous mutants when the plants carried a –r allele and *Pl'*. This was the case for the *rmr1-1* families and for approximately ¼ of the *Mop1-1* families. Crosses with *Mop1-1* testers confirmed that plants that failed to activate the transgene were heterozygous for the mutation. For the experiments with *rmr2-1* a dominant *r1* allele was segregating, which also conferred purple aleurone pigment. Thus, planting purple kernels did not ensure that all plants would have inherited the transgene. As *r1* segregated independently of the transgene and rmr2, ¼ of the plants would be expected to receive the transgene and be homozygous for *rmr2-1*. The numbers of plants with an active transgene were consistent with this hypothesis (Table 12). Independent of whether we could use anther color scores to assess whether the mutation was heterozygous or homozygous, every plant was crossed with appropriate mutant testers to determine or confirm the mutant genotype. Not all crosses were successful, but in all confirmed cases with *Mop1-1* and *rmr1-1*, when the mutation was homozygous, the

transgene was activated as evidenced by increased pigment in homozygous mutant plants relative to heterozygous siblings. One possible exception is indicated by the significant deviation from the expected 1:1 ratio in the *Mop2-1* experiment. We have considered two possible explanations for this deviation from the expected ratios. First, it is possible that homozygous *Mop2-1* individuals are not always capable of activating the transgene. It is also possible that the penetrance of the *Mop2-1* phenotype in these families was low. Testcrosses are in progress to determine the genotype of plants in each class. The *Mop2-1* mutation was also crossed with an independent 35S*B-l*genomic transgenic line (VLC44 –20). This transgene was expressed in both the plant and seed for several generations, but silenced in the generation immediately prior to crossing with *Mop2-1*. When this transgene was present with heterozygous *Mop2-1* it was not activated, but when the F1 was crossed with a homozygous *Mop2-1* plant, 4/9 of the progeny activated the transgene (same phenotype as seen in Figure 29A).

[00327] **Transgene activation involves increases in the transgene RNA.** Based on the effect of the mutants on *B'* and *Pl'* RNA levels, we hypothesized that the increased pigment levels seen in the transgenic lines containing the homozygous mutations was due to increased levels of RNA from the transgene. To investigate this we took samples from families carrying the VLC44-27A transgene and segregating individuals heterozygous or homozygous for several mutations. RNA was prepared using the same procedure as described in Example 1, and RNA blots prepared. As shown in Figure 30, each of the darkly pigmented plants (homozygous for either *Mop1-1*, *rmr1-1* or *rmr2-1*) had dramatic increases in transgene-encoded *b1* RNA.

[00328] **The transgene is activated at the transcriptional level.** To investigate whether the increased *b1* RNA levels are caused by increases in transcription or increases in RNA stability we have begun to perform nuclear run-on assays (methods as described in Example 1). One set of experiments have been completed for *Mop1-1* and *rmr2-1* (Figure 31). These initial results reveal that the VLC44-27A transgene (35S*B-l*gen) is transcribed at a low level in plants heterozygous for either *Mop1-1* or *rmr2-1* (light plants), and this is dramatically increased in plants homozygous for *Mop1-1* or *rmr2-1* (dark plants). This result was observed for both the *b1* transcript and for the vector backbone (+SK) used in the original construct. The latter result indicates that the vector backbone is being transcribed. The VCL44-27A transgene contains multiple rearranged copies (Y. Lin and V. Chandler, unpublished data), which is clearly enabling transcription of the vector sequences to occur, presumably due to juxtaposition near a promoter.

[00329] **The transgene can remain active when *Mop1-1* and *rmr2-1* are segregated away.** We next tested whether the transgene could remain active in progeny plants that were again heterozygous for the mutations. For this experiment multiple individual plants that contained the active 35S*B-I*gen transgene and were homozygous for *Mop1-1*, *rmr1-1* or *rmr2-1* were outcrossed to b *R-g Pl*stocks, which contained dominant functional alleles for all three genes (*Mop1*, rm*r1* and rmr2). Purple seed, which contained the transgene were planted and the resulting plants scored for transgene pigmentation. With *rmr1-1*, all of the resulting plants were green, indicating that the transgene was efficiently resilenced in the presence of a functional Rm*r1* allele. For *Mop1-1* and *rmr2-1*, the transgene was resilenced in most of the progeny, but there were exceptions, indicating that in rare individuals the transgene could remain active, even in the presence of functional *Mop1* or Rmr2 alleles. The number of plants that produced progeny with an active transgene and the total number tested are summarized for each mutation in Table 13.

Table 13. Heritability of the active transgene in heterozygous plants

	Mop1-1	*rmr1-1*	*rmr2-1*
# homozygous plants outcrossed to wildtype	9	33	8
# with dark progeny	4	0	5
# total progeny scored	183	590	198
# dark plants	15	0	21

[00330] We then tested whether the transgene that remained active in *Mop1/Mop1-1* or Rmr2/*rmr2-1* plants would remain active into the next generation by crossing these again to b *R-g Pl*testers. One-half of the progeny would be expected to be heterozygous for *Mop1-1* or *rmr2-1* and the other half homozygous wild type. Seeds containing the transgene were identified by their seed pigmentation, planted and scored for transgene expression in the plant. A total of 6 and 12 plants from the *Mop1* and rmr2 experiments were tested, respectively (Table 14). Six of the six *Mop1/Mop1-1* heterozygotes and 11/12 of the Rmr2/*rmr2-1* heterozygotes transmitted active transgenes to most if not all of their progeny (Table 14). Interestingly, in this last generation there was a much larger number of progeny plants that carried an active transgene, as compared to the first generation of crosses (compare Table 13 and Table 14), indicating that the transgene was heritably activated and it remains active in subsequent generations in the absence of the mutations that induced the original activation. Thus, it may be possible to use these mutations to activate other silent transgenes and then segregate the mutations from the active transgene. Additional crosses are in progress to determine the stability of the active transgenes in the absence of the mutations.

Table 14. Heritability of the active transgene in wildtype plants.

	Mop1-1	*rmr2-1**
# heterozygous plants outcrossed to wildtype	6	12
# with dark progeny	6	11
# total progeny scored	390	567
# dark plants	338	333

[00331] *A functional *r1* allele that pigments the seed is segregating in these crosses, such that not all plants with purple seed will carry the transgene.

[00332] **Summary**. Our results demonstrate that four different genes that are required for paramutation (*Mop1*, mop2, rm*r1* and rmr2) are also required to maintain the silent state of several transgene loci. Our initial results with mutations in two genes (*Mop1-1* and *rmr2-1*) suggest that the activation is occurring at the transcriptional level. Our experiments further demonstrate that the *Mop1-1* and *rmr2-1* mutations can heritably activate the transgene, such that it remains active even when the mutations are segregated away. Additional experiments need to be done to determine the stability of the transcriptional activation and to determine if the other two mutations, *Mop2-1* and *rmr1-1* also activate the transgene at the transcriptional level. While all of the transgenes tested contain part of the *b1* gene, none of the transgenes contain the sequences required for *b1* paramutation (M. Stam and V. Chandler, unpublished data). Thus, we hypothesize that the activation of the *b1* transgenes by these mutations will be a general effect that will occur with other transgenes as well. This is currently being tested using several transgenes that contain no *b1* sequences. Additional experiments will also be required to determine if these mutations can activate transgenes that are post-transcriptionally silenced.

Example 6

Identification and characterization of additional recessive mutations that reduce the maintenance of *pl1* paramutation

[00333] **Overview**. Additional maize mutations that reduce the maintenance of the *Pl'* paramutant state were identified from seedling screens similar to those described in Example 3 and mature plant screens using EMS-derived materials described in Example 3. Genetic complementation tests suggest that these mutations define five novel rmr loci (rmr6, rmr7, rmr8, rmr9, rm*r11*) and two new alleles of *Mop1*. RMR6 is required for transcriptional repression of *Pl'*. RMR6, RMR9 and RM*R11* functions are required to heritably maintain the *Pl'* paramutant state. Phenotypic analysis indicates that RMR6, RMR8, and RM*R11* functions are important for proper plant growth and development. Mutant corn plants that are

homozygous for mutations in either the rmr7 and rmr9 genes have a normal developmental phenotype.

1. **Materials and Methods:**

[00334] See Example 3.

2. **Identification and characterization of the rmr6 locus.**

[00335] **Description.** Seedling screening similar to that described in Example 3 was used to identify an M2 family (96406) that segregated darkly pigmented seedlings. The genetic factor responsible for this darkly pigmented seedling phenotype is inherited as a single locus recessive factor that is not closely linked to the *pl1* locus (see below). Genetic complementation test results (see below) demonstrate that this recessive factor defines a novel locus designated required to maintain repression 6 (rmr6) whose function is needed to maintain transcriptional repression of *Pl'* and sexual transmission of the *Pl'* paramutant state at a 100% frequency. The recessive rmr6 mutation identified by the seedling screen is designated *rmr6-1*.

[00336] ***rmr6-1* is inherited as a single locus recessive mutation.** Homozygous *rmr6-1/rmr6-1* plants having a *Pl-Rh*-like phenotype were outcrossed to *Pl'/Pl'* testers. All F1 progeny plants (32 individuals from 5 independent outcrosses) had a *Pl'* anther phenotype (7 ACS 1; 17 ACS 2; 8 ACS 3) indicating that the *rmr6-1* allele is recessive and further that *Pl'* alleles transmitted from homozygous *rmr6-1/rmr6-1* plants are not recalcitrant to subsequent paramutation. The *Pl-Rh*-like phenotype was recovered in 21/123 (17%) F2 plants derived from three independent self-pollinations of F1 plants (44 ACS 1; 46 ACS 2; 12 ACS 3; 21 ACS 7). The observed frequency of mutant phenotypes (17%) is not significantly different from the 25% expected from the segregation of a single locus recessive mutation ($\chi^2 = 0.32$; $P > 0.05$).

[00337] ***rmr6-1* defines a trans-acting function acting upon Pl.** The mutant phenotype (*Pl-Rh*-like anthers) did not cosegregate with alleles of salmon silks1 (sm1), a genetic locus 10 cM distal to the *pl1* locus. The *Pl-Rh* allele subjected to EMS mutagenesis is genetically linked to the recessive sm1 allele sm1-EMS, therefore any mutations that occur close to the *pl1* locus should also be genetically linked to sm1-EMS. One of the F2 families described above had individuals that were either wild type or mutant with regards to the salmon silks trait. Two of 43 (5%) of the wild type plants (Sm1/Sm1 or Sm1/sm1-EMS) had a *Pl-Rh*-like phenotype and 3 of 11 (27%) of the mutant (sm1-EMS/sm1-EMS) plants had a *Pl-Rh*-like phenotype. The absence of strong cosegregation between the *Pl-Rh*-like phenotypes and

mutant sm1 phenotypes indicates that *rmr6-1* is not tightly linked to sm1 and therefore not tightly linked to *pl1*. This result strongly suggests that the rmr6 locus is distinct from *pl1*.

[00338] ***rmr6-1* defines a novel rmr locus.** Complementation crosses were made between homozygous mutant plants (*Pl-Rh* anthers) and plants heterozygous for each different mutation (*Pl'* anthers). All tests completed to date are summarized in Table 15. The *rmr6-1* mutation clearly complemented all the mutations, except *rmr11-1*. If *rmr6-1* and *rmr11-1* were alleles of the same gene, we would expect that 1/2 of the complementation test progeny (~26 individuals) would have *Pl-Rh*-like anthers. Our observed frequency of *Pl-Rh*-like phenotypes (15/52) is significantly different from this expectation ($\chi^2 = 5$; $P < 0.05$). Given that the *rmr11-1* mutation also shows non-complementation with the *rmr1-1* mutation, the most likely interpretation of these results is that *rmr6-1* defines a locus distinct from rm*r11*. However, the observed partial non-complementation suggests that RMR6 and RM*R11* functions may cooperate to achieve efficient repression of *Pl'*.

TABLE 15
Complementation Tests

Alleles Tested	Ears Examined	Number of Plants with Given Anther Color Score						
		1	2	3	4	5	6	7
rmr6-1								
rmr1-1	4	19	35	28	5	1	1	0
rmr2-1	4	14	38	35	1	0	0	0
Mop1-1	3	22	27	6	1	0	0	0
rmr7-1	2	13	10	2	0	0	0	0
rmr7-2	2	11	18	2	0	0	0	0
rmr8-1	2	4	22	3	0	0	0	0
rmr9-1	2	1	16	10	0	0	0	0
rmr11-1	3	1	13	17	6	1	0	15
Mop1-4	2	1	16	10	0	0	0	0
Mop1-5	2	7	15	2	1	0	0	0
rmr7-1								
rmr1-1	3	16	16	3	0	0	0	0
rmr2-1	2	12	13	4	0	0	0	0
Mop1-1	3	23	10	2	0	0	0	0
Mop1-4	2	21	12	3	0	0	0	0
Mop1-5	1	10	11	0	1	0	0	0
rmr7-2	2	6	8	1	1	5	11	5
rmr8-1	1	20	28	0	0	0	0	0
rmr9-1	2	12	14	2	0	0	0	0

rmr7-2								
rmr1-1	2	19	11	0	0	0	0	0
rmr2-1	2	11	23	1	1	0	0	0
Mop1-1	2	18	13	1	0	0	0	0
Mop1-4	2	13	19	5	0	0	0	0
rmr8-1	2	5	18	3	1	0	0	0
rmr9-1	2	3	33	1	0	0	0	0

rmr8-1								
rmr1-1	3	6	11	5	7	5	1	0
rmr2-1	1	0	1	10	2	0	0	0
Mop1-2EMS	2	0	9	7	7	5	3	2
Mop1-4	1	2	7	5	5	1	1	1
Mop1-5	2	9	16	2	0	0	0	0
rmr9-1	3	6	11	4	10	13	20	6

rmr9-1								
rmr1-1	3	4	12	13	0	0	0	0
rmr2-1	2	1	6	21	1	0	0	0
Mop1-2EMS	2	1	8	19	1	0	0	0
Mop1-4	1	4	21	1	0	0	0	0
Mop1-5	2	10	16	5	1	0	0	0
rmr11-1	1	1	3	6	0	0	0	0

rmr11-1								
rmr1-1	3	11	20	2	2	0	1	37
rmr2-1	1	13	13	0	0	0	0	0
Mop1-4	1	10	7	4	0	0	0	0

Mop1-4								
rmr1-1	2	4	19	17	0	0	0	0
rmr2-1	1	2	14	10	0	0	0	0
Mop1-1	2	8	8	5	0	0	0	8
Mop1-5	2	1	14	1	2	0	0	8

Mop1-5								
rmr1-1	2	1	12	1	1	0	0	0
rmr2-1	2	10	11	2	4	0	0	0
Mop1-1	2	5	1	1	0	0	0	12

[00339] Complementation tests identify four unique loci. For each allele listed in bold, the underlying series of alleles were tested for complementation. The number of ears sampled for each test are indicated along with the total numbers of mature plants with a given Anther Color Score derived from the seeds off those ears. Complementation should result in all plants with an ACS between 1-5. This result is consistent with the mutations being in different genes. Failure to complement would produce 50% darkly pigmented individuals (ACS 6-7). The simplest interpretation of this result is that the two mutations are in the same gene. However, it is also possible that when two genes are acting on the same pathway,

double heterozygotes can show failure to complement. This is well documented and referred to as extragenic noncomplementation.

[00340] ***Pl'* can change back to *Pl-Rh* in homozygous *rmr6-1/rmr6-1* plantS.** Crosses were made to four different homozygous *Pl-Rh* tester stocks to determine if all the *pl1* alleles sexually transmitted from *Pl'/Pl'*; *rmr6-1/rmr6-1* plants remained paramutagenic. Normally, crosses between *Pl-Rh/Pl-Rh* testers and *Pl'/Pl'* plants give rise exclusively to progeny with a *Pl'/Pl'* phenotype and genotype (Hollick et al. 1995). Twenty-five out of 36 crosses made between *Pl-Rh/Pl-Rh* testers and *Pl'/Pl'*; *rmr6-1/rmr6-1* plants gave rise to plants with *Pl-Rh*-like anthers. In total, 167/688 (24%) of the testcross progeny had ACS7 anthers (73 ACS 1; 232 ACS 2; 117 ACS 3; 50 ACS 4; 32 ACS 5; 17 ACS 6; 167 ACS 7). Thus *Pl'* alleles can change back to a meiotically-heritable, non-paramutagenic, *Pl-Rh* state in plants homozygous for *rmr6-1*.

[00341] **The *rmr6-1* mutation affects plant development.** The *Pl-Rh*-like phenotype seen in homozygous *rmr6-1* plants cosegregates with uniform alterations of normal plant development. First, homozygous *rmr6-1* plants flower 5-7 days later, on average, than their heterozygous siblings. Second, homozygous *rmr6-1* plants are 25% shorter, on average, than their heterozygous siblings. Third, homozygous *rmr6-1* plants resemble tiller shoots in that there is frequent feminization of the apical inflorescence (Figure 35), retardation of internode elongation for the apical leaves, and poor ear shoot development including the occurrence of disorganized rows of grains on the mature ears. Pollen grains from homozygous *rmr6-1* plants appear visibly normal although no fertility tests have been conducted. These morphological phenotypes have been seen in all *rmr6-1* families tested to date including introgression material using the maize elite inbred A632.

[00342] **The rmr6 gene product is required for transcriptional repression of the *Pl'* allele.** RNase protection experiments like those described in Examples 1 and 3 indicate that *pl1* RNA levels are 26-fold higher in anther tissues of homozygous *rmr6-1* plants relative to their heterozygous siblings (Figure 36A). Five independent in vitro transcription assays (see Example 1 for methods) using isolated husk nuclei demonstrate that *pl1* transcription is 4-fold higher in homozygous *rmr6-1* plants relative to their heterozygous siblings (Figure 36B-C). Thus RMR6 function is required to maintain transcriptional repression of the *Pl'* allele.

[00343] **RMR6 is required to maintain cytosine methylation at *Mutator* terminal inverted repeats.** Genomic DNA samples from a family of individuals segregating 1:1 for *rmr6-1/rmr6-1* and *rmr6-1/+* types was analyzed for the cytosine methylation status of *Mu1* sequences as described in Example 2 above. All five of the *rmr6-1/+* plants had

hypermethylated *Mu1* terminal inverted repeats, whereas six of the seven *rmr6-1/rmr6-1* plants had hypomethylated *Mu1* terminal inverted repeats (Figure 34A). Thus RMR6 function appears to be involved in maintaining the hypermethylated state of *Mu1* terminal inverted repeat sequences.

4. Identification and characterization of the rmr7 locus.

[00344] **Description.** Seedling screens similar to that described in Example 3 were used to identify two M2 families (9650 and 98939) that segregated darkly pigmented seedlings. The genetic factors responsible for these darkly pigmented seedling phenotypes are inherited as single locus recessive factors that are not genetically linked to the *pl1* locus (see below). Genetic complementation test results (Table 15) demonstrate that these two recessive factors define a novel locus designated required to maintain repression 7 (rmr7). The recessive rmr7 mutations identified by the seedling screens are designated *rmr7-1* (formerly 9650) and *rmr7-2* (formerly 98939).

[00345] ***rmr7-1* is inherited as a single locus recessive mutation.** Homozygous *rmr7-1/rmr7-1* plants having a *Pl-Rh*-like phenotype were outcrossed to *Pl'/Pl'* testers. All the F1 progeny plants (22 individuals from 2 independent outcrosses) had a *Pl'* anther phenotype indicating that the *rmr7-1* allele is recessive and further that *Pl'* alleles transmitted from homozygous *rmr7-1/rmr7-1* plants are still capable of causing paramutation. The *Pl-Rh*-like phenotype was recovered in 34/154 (22%) F2 plants derived from seven independent self-pollinations of F1 plants (120 ACS 1-ACS 4; 34 ACS 7). The observed frequency of mutant phenotypes (22%) is not significantly different from the 25% expected from a single locus recessive mutation ($\chi^2 = 0.53$; $P > 0.05$).

[00346] ***rmr7-2* is transmitted as a recessive mutation.** Homozygous *rmr7-2/rmr7-2* plants having a *Pl-Rh*-like phenotype were outcrossed to *Pl'/Pl'* testers. All F1 progeny plants (23 individuals from 2 independent outcrosses) had a clear *Pl'* anther phenotype indicating that the *rmr7-2* allele is recessive and further that *Pl'* alleles transmitted from homozygous *rmr7-2/rmr7-2* plants are not recalcitrant to subsequent paramutation.

[00347] ***rmr7-1* defines a trans-acting function acting upon Pl.** The *Pl-Rh*-like anther phenotype conferred in *rmr7-1/rmr7-1* plants did not co-segregate with the *pl1* locus in a F2 mapping population. The F2 mapping population was derived by first crossing a *Pl'/Pl'*; *rmr7-1/rmr7-1* plant to the elite inbred A632 (pl-A632/pl-A632; Rmr7-A632/Rmr7-A632) and then self-pollinating one of the resultant F1 plants. Fifty-six of 189 (30%) F2 plants were Pl/*Pl*based on linked simple satellite repeat (SSR) polymorphisms yet only ten of these 56

Pl/*Pl*plants (18%) had a *Pl-Rh*-like anther phenotype (10 ACS 7). If the *rmr7-1* mutation was tightly linked to the *Pl'* allele then the expectation was that greater than 50% of the Pl/*Pl*F2 plants would also have a dark-anther phenotype. The absence of co-segregation between the Pl/*Pl*genotype and the *Pl-Rh*-like phenotype indicates that the rmr7 locus is unlinked to the *pl1* locus. All 46 pl-A632/pl-A632 plants had visibly similar levels of anther pigment suggesting that RMR7 function specifically affects expression of the *Pl'* allele and is not a general modifier of other *pl1* alleles or of anthocyanin production in general.

[00348] ***rmr7-1* and *rmr7-2* define a novel rmr locus.** Complementation crosses were made between homozygous mutant (*Pl-Rh* anthers) plants and plants heterozygous for a different mutation (*Pl'* anthers). Results of these complementation crosses are shown in Table 15. The *rmr7-1* and *rmr7-2* mutations fail to complement each other indicating that these two mutations define alleles of the same gene. Other test results demonstrate that the *rmr7-1* mutation complements mutations at rmr*1*, rmr2, *Mop1*, rmr6, rmr8, and rmr9. Thus the *rmr7-1* and *rmr7-2* mutations identify a novel rmr locus, required to maintain repression 7.

[00349] ***Pl'* does not change back to a meiotically-heritable *Pl-Rh* state in homozygous *rmr7-1*or *rmr7-2* plants.** In wild type backgrounds, crosses between *Pl-Rh*/*Pl-Rh* testers and *Pl'*/*Pl'* plants give rise exclusively to progeny with a *Pl'*/*Pl'* phenotype and genotype (Hollick et al. 1995). However, *Pl'* can change back to *Pl-Rh* at particular frequencies when carried with several of the other homozygous mutants (*Mop1-1*, Dorweiler et al, 2000; *rmr1-1*, *rmr1-2*, *rmr2-1*, Hollick and Chandler 2001; and *rmr6-1*, *rmr9-1*, *rmr11-1*, this document). To determine if all the *pl1* alleles sexually transmitted from *Pl'*/*Pl'*; *rmr7-1*/*rmr7-1* plants remained paramutagenic, crosses were made to seven different homozygous *Pl-Rh* tester stocks. None of the 12 crosses made between *Pl-Rh*/*Pl-Rh* testers and *Pl'*/*Pl'*; *rmr7-1*/*rmr7-1* plants gave rise to progeny plants with *Pl-Rh*-like anthers. The distribution of anther color scores among the progeny was (119/235 ACS 1; 100/235 ACS 2; 12/235 ACS 3; 4/235 ACS 4). Similarly, crosses were made to four different homozygous *Pl-Rh* tester stocks to determine if all the *pl1* alleles sexually transmitted from *Pl'*/*Pl'*; *rmr7-2*/*rmr7-2* plants remained paramutagenic. None of the 4 crosses made between *Pl-Rh*/*Pl-Rh* testers and *Pl'*/*Pl'*; *rmr7-2*/*rmr7-2* plants gave rise to progeny plants with *Pl-Rh*-like anthers. The distribution of anther color scores among the progeny was (25/69 ACS 1; 39/69 ACS 2; 5/69 ACS 4). These anther color scores are very similar to that seen in the progenies of crosses between *Pl'*/*Pl'* (wild type for all paramutation defect loci) and *Pl-Rh*/*Pl-Rh* plants (Hollick et al. 1995). Thus, there is no evidence that lack of RMR7 function allows *Pl'* to change back to a meiotically-heritable *Pl-Rh* state.

[00350] **The *rmr7-1* and *rmr7-2* mutations do not affect plant development.** The *Pl-Rh*-like phenotype seen in homozygous *rmr7-1* and homozygous *rmr7-2* plants is not associated with any obvious developmental abnormalities. Field observations of F2 families segregating either the *rmr7-1* or *rmr7-2* mutations as well as comparison of *rmr7-1/rmr7-1* and *rmr7-1/+* individuals derived from intercrossing *rmr7-1/rmr7-1* and *rmr7-1/+* F2 siblings show relative uniformity of plant morphology. There are currently no observations to indicate that RMR7 functions are required for normal growth and development.

5. Identification and characterization of the rmr8 locus.

[00351] **Description.** This locus was identified from screening mature plant phenotypes of M2 families derived from the original chemical mutagenesis described in Example 3. Mature plants with full-colored anthers were found segregating in this particular M2 family (9583). This mutation does not affect the seedling pigment phenotype of *Pl'* plants; its effects are only manifest in the main body of the plant and in the anthers. Homozygous *rmr8-1/rmr8-1, Pl'/Pl'* seedlings have weak coloration indistinguishable from that of *Pl'/Pl'* seedlings but the anthers of *rmr8-1/rmr8-1, Pl'/Pl'* plants are indistinguishable from those of *Pl-Rh/Pl-Rh* plants. The genetic factor responsible for this darkly pigmented anther phenotype is inherited as a single locus recessive factor that is genetically unlinked to the *pl1* locus (see below). Genetic complementation test results (Table 15) demonstrate that this recessive factor defines a novel locus designated required to maintain repression 8 (rmr8). The recessive rmr8 mutation identified by the mature plant screen is designated *rmr8-1*.

[00352] ***rmr8-1* is inherited as a single locus recessive mutation.** Homozygous *rmr8-1/rmr8-1* plants having a *Pl-Rh*-like phenotype were outcrossed to *Pl'/Pl'* testers. All F1 progeny plants (29 individuals from 2 independent outcrosses) had a clear *Pl'* anther phenotype indicating that the *rmr8-1* allele is recessive and further that *Pl'* alleles transmitted from homozygous *rmr8-1/rmr8-1* plants are still capable of paramutation. The dark-anther phenotype (ACS 5, 6, and 7 classes) was recovered in 7/54(13%) F2 plants derived from three independent self-pollinations of F1 plants (3 ACS 1; 18 ACS 2; 21 ACS 3; 5 ACS 4; 2 ACS 5; 2 ACS 6; 3 ACS 7). The observed frequency of non-*Pl'* phenotypes (13%) is significantly different from the 25% expected from a single locus recessive mutation (χ^2 = 3.13; $P < 0.05$) and is also significantly different from the 6.25% expected if the dark-anther trait is due to the presence of two unlinked recessive mutations (χ^2 = 3.89; $P < 0.05$). It is possible that the low frequency of dark-anthered plants in these F2 families was due to lethal effects of an EMS-induced mutation found closely linked to rmr8. In support of this hypothesis, segregation ratios more closely approximated 25% in subsequent F2 families derived from advanced generations of *rmr8-1/rmr8-1* material. Four of 17 (24%) and 33/119

(28%) F2 plants from two independent families had dark-anther phenotypes (combination of ACS 5, 6 and 7) consistent with the hypothesis that the dark-anther phenotype is due to a single locus recessive mutation.

[00353] ***rmr8-1* defines a trans-acting function affecting anther pigmentation.** The dark-anther phenotype conferred in *rmr8-1/rmr8-1* plants did not co-segregate with the *pl1* locus in a F2 mapping population. The F2 mapping population was derived by first crossing a *Pl'/Pl'*; *rmr8-1/rmr8-1* plant to the elite inbred A632 (pl-A632/pl-A632; Rmr8-A632/Rmr8-A632) and then self-pollinating one of the resultant F1 plants. Forty-one of 169 (24%) F2 plants were Pl/*Pl*based on linked simple satellite repeat (SSR) polymorphisms yet only six of these 41 Pl/*Pl*plants (15%) had a dark-anther phenotype (1 ACS 5; 1 ACS 6; 4 ACS 7). If the *rmr8-1* mutation was linked to the *Pl'* allele then the expectation was that greater than 50% of the Pl/*Pl*F2 plants would also have a dark-anther phenotype. The absence of co-segregation between the Pl/*Pl*genotype and the dark-anther phenotype indicates that the rmr8 locus is unlinked to the *pl1* locus. Eleven of 46 pl-A632/pl-A632 plants (24%) had visibly more anther pigment than the remaining pl-A632/pl-A632 siblings. This is the frequency of individuals expected to carried be homozygous for *rmr8-1* allele, suggesting that RMR8 function might increase pigment on other *pl1* alleles in addition to the *Pl'* allele (see below).

[00354] ***rmr8-1* defines a novel rmr locus.** Complementation crosses were made between homozygous mutant (*Pl-Rh* anthers) plants and plants heterozygous for a different mutation (*Pl'* anthers). Results shown in Table 15 indicate that the *rmr8-1* mutation complements the *rmr1-1*, *rmr2-1*, *rmr6-1*, *rmr7-1*, and *Mop1-5* mutations, suggesting it is at a distinct locus. However, *rmr8-1* showed partial complementation with the *rmr9-1*, *Mop1-2EMS*, and *Mop1-4* mutations. If the *rmr8-1* mutation is an allele of *Mop1* the expectation is that all alleles of *Mop1* should behave similarly. This was not observed. No dark anthered individuals were observed in progeny from the *Mop1-5* experiments, and much fewer than the predicted 50% were observed in progeny from the *Mop1-4* experiment (Table 15). Only 1/22 progeny from this cross had a *Pl-Rh*-like anther phenotype (2 ACS 1; 7 ACS 2; 5 ACS 3; 5 ACS 4; 1 ACS 5; 1 ACS 6; 1 ACS 7) and only 3/22 progeny had a dark-anther phenotype (ACS 5, 6 or 7). Finally, the observed frequency of *Pl-Rh*-like types (ACS 7) in the *Mop1-2EMS* progeny is significantly different from the frequency expected for this hypothesis (□2=12.25; $P < 0.01$), but the observed frequency of dark-anther phenotypes (ACS 5, 6 or 7) is not significantly different from 50% (□2=2.25). The most likely interpretation of these results is that *rmr8-1* and several *Mop1* alleles show partial non-complementation and *rmr8-1* defines a locus distinct from *Mop1*. The observed partial non-complementation suggests that RMR8 and *Mop1* functions may cooperate to achieve efficient repression of *Pl'*.

[00355] If the *rmr8-1* mutation is an allele of rmr9, then 1/2 of all progeny (35 individuals) from these two crosses should have a *Pl-Rh*-like anther phenotype. The observed frequency of *Pl-Rh*-like types is significantly different from the expected frequency for this hypothesis (χ^2=24; P < 0.01). However, the observed frequency of dark-anther phenotypes (ACS 5, 6 or 7) is not significantly different from 50% (χ^2=0.71; P > 0.05). Given that the *rmr9-1* mutation shows full complementation with the *Mop1-2EMS* and *Mop1-4* mutations (Table 15), while the *rmr8-1* mutation partially complements these mutations, the most likely interpretation of these results is that *rmr8-1* defines a locus distinct from rmr9. The observed partial non-complementation between *rmr8-1* and *rmr9-1* suggests that RMR8 and RMR9 functions may cooperate to achieve efficient repression of *Pl'*. It is interesting that *rmr8-1* interacts with both *rmr9-1* and several *Mop1* mutations, but there is no observed interaction in the complementation tests between *rmr9-1* and the *Mop1* mutations.

[00356] ***Pl'* does not change back to a meiotically-heritable *Pl-Rh* state in homozygous *rmr8-1/rmr8-1* plants**. Crosses were made to four different homozygous *Pl-Rh* tester stocks to determine if all the *pl1* alleles sexually transmitted from *Pl'/Pl'*; *rmr8-1/rmr8-1* plants remained paramutagenic, which occurs in wild type stocks (Hollick et al. 1995). None of the 4 crosses made between *Pl-Rh/Pl-Rh* testers and *Pl'/Pl'*; *rmr8-1/rmr8-1* plants gave rise to progeny plants with *Pl-Rh*-like anthers. The distribution of anther color scores among the progeny (9/86 ACS 1; 51/86 ACS 2; 13/86 ACS 3; 3/86 ACS 4) is very similar to that seen in the progenies of crosses between *Pl'/Pl'* in wild type stocks and *Pl-Rh/Pl-Rh* plants (Hollick et al. 1995). Thus, there is no evidence that lack of RMR8 function allows *Pl'* to change back to a meiotically-heritable *Pl-Rh* state.

[00357] **The *rmr8-1* mutation affects plant development**. The *Pl-Rh*-like phenotype seen in homozygous *rmr8-1* plants co-segregates with uniform developmental abnormalities. Homozygous *rmr8-1* plants have a smaller tassel with reduced numbers of tassel branches, failure of anther extrusion from the glumes, and a limited development of female reproductive structures in the tassel. The severity of this suite of traits is fairly consistent and all the plants with darkly pigmented anthers clearly have the same suite of developmental abnormalities. The severity of these abnormal phenotypes remains constant in advanced generations (3 generations of self-pollination) of a homozygous *rmr8-1* background.

[00358] **The rmr8 gene product is required for repression of *Pl'* expression**. A single RNase protection experiment like those described in Examples 1 and 3 indicate that *pl1* RNA levels are 11-fold higher in anther tissues of homozygous *rmr8-1* plants relative to their heterozygous siblings. It unknown if this effect on *pl1* RNA levels is controlled at the

transcriptional or post-transcriptional level. Nonetheless, RMR8 function is required to maintain repression of the *Pl'* allele.

6. Identification and characterization of the rmr9 locus

[00359] **Description**. This locus was identified from screening mature plant phenotypes of M2 families derived from the original chemical mutagenesis described in Example 3. Mature plants with full-colored anthers were found segregating in this particular M2 family (95270). This mutation does not affect the seedling pigment phenotype of *Pl'* plants; its effects are only manifest in the main body of the plant and in the anthers. Homozygous *rmr9-1/rmr9-1*, *Pl'/Pl'* seedlings have weak coloration indistinguishable from that of *Pl'/Pl'* seedlings, while the anthers of *rmr9-1/rmr9-1*, *Pl'/Pl'* plants are indistinguishable from those of *Pl-Rh/Pl-Rh* plants. The genetic factor responsible for this darkly pigmented anther phenotype is inherited as a single locus recessive factor that is genetically unlinked to the *pl1* locus (see below). Genetic complementation test results (Table 15) suggest that this recessive factor defines a novel locus designated required to maintain repression 9 (rmr9) whose function is required to maintain sexual transmission of the *Pl'* paramutant state at 100% frequency. The recessive rmr9 mutation identified by the mature plant screen is designated *rmr9-1*.

[00360] ***rmr9-1* is inherited as a single locus recessive mutation**. Homozygous *rmr9-1/rmr9-1* plants having a *Pl-Rh*-like phenotype were outcrossed to *Pl'/Pl'* testers. All F1 progeny plants (23 individuals from a single outcross) had a *Pl'* anther phenotype indicating that the *rmr9-1* allele is recessive and further that *Pl'* alleles transmitted from homozygous *rmr9-1/rmr9-1* plants are not recalcitrant to subsequent paramutation. The *Pl-Rh*-like anther phenotype (ACS 7) was recovered in only 2/29 (7%) F2 plants derived from three independent self-pollinations of F1 plants (3 ACS 1; 12 ACS 2; 6 ACS 3; 6 ACS 4; 2 ACS 7). The observed frequency of *Pl-Rh*-like phenotypes (7%) is significantly different from the 25% expected from a single locus recessive mutation ($\chi^2 = 3.8$; $P < 0.05$), but is not significantly different from the 6.25% expected if the dark-anther trait is due to the presence of two unlinked recessive mutations ($\chi^2 = 0.02$; $P > 0.05$). It is also possible that the low frequency of dark-anther plants in these F2 families was due to lethal effects of an EMS-induced mutation found closely linked to rmr9. In support of this latter hypothesis, segregation ratios more closely approximated 25% in subsequent F2 families derived from advanced generation *rmr9-1/rmr9-1* material. Three of 16 (19%) and 5/22 (23%) F2 plants from two independent families had *Pl-Rh*-like anther phenotypes. These latter segregation ratios are more consistent with the single locus recessive hypothesis ($\chi^2 = 0.25$ and $\chi^2 = 0.045$, respectively)

rather than a model in which the dark-anther trait is due to the presence of two unlinked recessive mutations ($\chi^2 = 4$; $P < 0.05$ and $\chi^2 = 9.6$; $P < 0.01$, respectively).

[00361] ***rmr9-1* defines a trans-acting function affecting anther pigmentation**. The dark-anther phenotype conferred in *rmr9-1/rmr9-1* plants did not co-segregate with specific *pl1* alleles in a F2 segregating family. To generate this particular F2 family, a *Pl'/Pl'*; *rmr9-1/rmr9-1* individual was first crossed to a *Pl'/Pl'* tester stock in which the *Pl'* alleles are linked (<1cM) to a translocation breakpoint between the long arm of chromosome 6 and the short arm of chromosome 9. This translocation breakpoint is also tightly linked to a recessive kernel mutation at the waxy1 locus (wx1) found on chromosome 9. Thus the *Pl'* alleles in these *Pl'/Pl'* testers are approximately 5cM from mutant wx1 alleles. Resulting F1 progeny had a clear *Pl'* phenotype and also displayed ~25% pollen abortion, which is expected in a translocation heterozygote. Upon self-pollination, the ears of these F1 plants segregated 1/4 waxy kernels as expected. Mutant wx1/wx1 kernels were selected and sowed. Three of the 16 plants grown from the wx1/wx1 kernels had a *Pl-Rh* (ACS 7) phenotype. If the *rmr9-1* mutation resides at the *pl1* locus, then none of the wx1/wx1 kernels should have also been homozygous for the *rmr9-1* mutation. Thus, preliminary data suggest that the rmr9 locus is distinct from *pl1*.

[00362] ***rmr9-1* defines a novel rmr locus**. Complementation crosses were made between homozygous mutant (*Pl-Rh* anthers) plants and plants heterozygous for a different mutation (*Pl'* anthers). Results (Table 15) indicate that the *rmr9-1* mutation complements mutations at rm*r1*, rmr2, *Mop1*, rmr6, rmr7 and rm*r11*. Thus the *rmr9-1* mutation identifies a new rmr locus, rmr9. The interactions with *rmr8-1* are discussed above.

[00363] ***Pl'* can change back to a meiotically-heritable *Pl-Rh* state in homozygous *rmr9-1/rmr9-1* plants**. Crosses were made to five different homozygous *Pl-Rh* tester stocks to determine if all the *pl1* alleles sexually transmitted from *Pl'/Pl'*; *rmr9-1*/rmr9 -1 plants remained paramutagenic as observed in wild type stocks (Hollick et al. 1995). One of the 5 crosses made between *Pl-Rh/Pl-Rh* testers and *Pl'/Pl'*; *rmr9-1/rmr9-1* plants gave rise to one progeny plant with *Pl-Rh*-like anthers. In addition, the distribution of Anther Color Scores among all the progeny of these 5 crosses (6/100 ACS 1; 42/100 ACS 2; 23/100 ACS 3; 18/100 ACS 4; 10/100 ACS 5; 1/100 ACS 7) is skewed towards higher Anther Color Scores than seen in the progenies of crosses between *Pl'/Pl'* and *Pl-Rh/Pl-Rh* plants wild type for RMR9 (Hollick et al. 1995). Thus, preliminary data suggest that *Pl'* alleles can sometimes change back to a meiotically-heritable, non-paramutagenic or weakly-paramutagenic state in plants homozygous for *rmr9-1*.

[00364] **The *rmr9-1* mutation does not affect plant development.** The *Pl-Rh*-like phenotype seen in homozygous *rmr9-1* plants is not associated with any obvious developmental abnormalities. Field observations of F2 families segregating the *rmr9-1* mutation as well as comparison of *rmr9-1/rmr9-1* and *rmr9-1/+* individuals derived from intercrossing *rmr9-1/rmr9-1* and *rmr9-1/+* F2 siblings show relative uniformity of plant morphology. There are currently no observations to indicate that RMR9 functions are required for normal growth and development.

7. Identification and characterization of the rm*r11* locus.

[00365] **Description.** Seedling screens similar to that described in Example 3 was used to identify an M2 family (98287) that segregated darkly pigmented seedlings. The genetic factor responsible for this darkly pigmented seedling phenotype is inherited as a single locus recessive factor that is not genetically linked to the *pl1* locus (see below). Genetic complementation test results (see below) suggest that this recessive factor defines a novel locus designated required to maintain repression 11 (rm*r11*), although this mutation appears to interact with mutations at several other loci. Further genetic tests indicate that RM*R11* function is required to maintain the *Pl'* paramutant state at 100% frequency through sexual transmission. The recessive rm*r11* mutation identified by the seedling screen is designated *rmr11-1*.

[00366] ***rmr11-1* is inherited as a single locus recessive mutation.** Homozygous *rmr11-1/rmr11-1* plants having a *Pl-Rh*-like phenotype were outcrossed to *Pl-Rh/Pl-Rh* testers. Almost 1/2 of all the F1 progeny plants (8 of 25 individuals from two independent outcrosses) had a *Pl-Rh* anther phenotype (2 ACS 1; 12 ACS 2; 2 ACS 4; 1 ACS 5; 8 ACS 7) suggesting that the *rmr11-1* allele might represent a dominant mutation. However, the *Pl-Rh*-like phenotype was recovered in 12/40 (30%) F2 plants derived from two independent self-pollinations of ACS 3 F1 plants (3 ACS 1; 19 ACS 2; 5 ACS 3; 1 ACS 4; 12 ACS 7). The observed frequency of F2 mutant phenotypes (30%) is not significantly different from the 25% expected from a single locus recessive mutation ($\chi^2 = 0.4$) and is consistent with the hypothesis that the dark-anther phenotype, is due to a single locus recessive mutation.

[00367] ***rmr11-1* defines a trans-acting function acting upon *Pl'*.** The mutant phenotype (*Pl-Rh*-like anthers) did not co-segregate with alleles of salmon silks1 (sm1), a genetic locus 10 cM distal to the *pl1* locus. The *Pl-Rh* allele that was subjected to EMS mutagenesis is genetically linked to the recessive sm1 allele, sm1-EMS. Therefore any mutations that occur close to the *pl1* locus should also be genetically linked to sm1-EMS. One of the F2 families mentioned above was segregating the salmon silks trait. Three of 17

F2 progeny had the recessive sm1-EMS/sm1-EMS phenotype yet none of these three plants had dark-colored anthers. Thus, the *rmr11-1* mutation appears to be genetically unlinked to the sm1 locus indicating that *rmr11-1* defines a genetic locus distinct from *pl1*.

[00368] ***rmr11-1* defines a novel rmr locus.** Complementation crosses were made between homozygous mutant (*Pl-Rh* anthers) plants and plants heterozygous for a different mutation (*Pl'* anthers). Results (Table 15) indicate that the *rmr11-1* mutation complements mutations at rmr2, rmr9 and *Mop1*. However, the *rmr11-1* mutation shows only partial complementation with the *rmr6-1* mutation and absence of complementation with the *rmr1-1* mutation. If the *rmr11-1* mutation was an allele of rmr*1*, then 1/2 of all progeny (37 individuals) from the three complementation crosses should have a *Pl-Rh*-like anther phenotype. The observed frequency of *Pl-Rh*-like types (37/83) is not significantly different from the expected frequency for this hypothesis (36.5/83), suggesting that the *rmr11-1* and *rmr1-1* mutations define the same locus. However, the *rmr11-1* mutation also shows partial non-complementation with the *rmr6-1* mutation, which is clearly a distinct locus from that defined by *rmr1-1*. Fifteen of the 53 progeny from the *rmr6-1* complementation crosses had a *Pl-Rh*-like anther phenotype (1 ACS 1; 13 ACS 2; 17 ACS 3; 6 ACS 4; 1 ACS 5; 15 ACS 7) demonstrating some non-complementation between the *rmr6-1* and *rmr11-1* mutations. If *rmr6-1* and *rmr11-1* were alleles of the same gene, we would expect that 1/2 of the progeny (26-27 individuals) would have *Pl-Rh*-like anthers. Our observed frequency of *Pl-Rh*-like phenotypes is significantly different from this expectation (χ^2=5; P < 0.05) indicating only partial genetic non-complementation. Given that the *rmr11-1* mutation fails to complement mutations previously shown to be non-allelic, the most likely interpretation of these results is that *rmr11-1* defines a locus distinct from either rmr*1* or rmr6.

[00369] ***Pl'* can change back to a meiotically-heritable *Pl-Rh* state in homozygous *rmr11-1*/*rmr11-1* plants.** Crosses were made to five different homozygous *Pl-Rh* tester stocks to determine if all the *pl1* alleles sexually transmitted from *Pl'*/*Pl'*; *rmr11-1*/*rmr11-1* plants remained paramutagenic as observed in wild type stocks (Hollick et al. 1995). Nine of the 15 crosses made between *Pl-Rh*/*Pl-Rh* testers and *Pl'*/*Pl'*; *rmr11-1*/*rmr11-1* plants gave rise to at least one progeny plant with *Pl-Rh*-like anthers (43/252 ACS 1; 105/252 ACS 2; 20/252 ACS 3; 21/252 ACS 4; 8/252 ACS 5; 3/252 ACS 6; 52/252 ACS 7). Thus, *Pl'* alleles can sometimes change back to a meiotically-heritable, non-paramutagenic or weakly-paramutagenic state in plants homozygous for *rmr11-1*.

[00370] **The *rmr11-1* mutation affects plant development.** The *Pl-Rh*-like phenotype seen in homozygous *rmr11-1* plants co-segregates with alterations of normal plant development. Homozygous *rmr11-1* plants resemble tiller shoots in that there is frequent

feminization of the apical inflorescence and there is poor ear shoot development, including the occurrence of disorganized rows of grains on the mature ears.

8. Identification and characterization of the *Mop1-4* and *Mop1-5* mutations

[00371] **Description**. Seedling screens similar to that described in Example 3 was used to identify two M2 families (98262 and 98941) that segregated darkly pigmented seedlings. The genetic factors responsible for these darkly pigmented seedling phenotypes are inherited as single locus recessive factors (see below). Genetic complementation test results (Table 15) suggest that these two recessive factors define additional alleles of *Mop1*. Further genetic tests indicate that these mutations can reduce the ability to maintain the *Pl'* paramutant state at a 100% frequency through sexual transmission. The recessive mutations identified by these seedling screens are currently designated *Mop1-4* and *Mop1-5*.

[00372] ***Mop1-4* is inherited as a single locus recessive mutation**. Homozygous *Mop1-4/Mop1-4* plants having a *Pl-Rh*-like phenotype were outcrossed to *Pl'/Pl'* testers. All F1 progeny plants (39 individuals from 3 independent outcrosses) had a *Pl'* anther phenotype (5 ACS 1; 22 ACS 2; 12 ACS 3) indicating that the *Mop1-4* allele is recessive and further that *Pl'* alleles transmitted from homozygous *Mop1-4/Mop1-4* plants are capable of paramutation. The *Pl-Rh*-like anther phenotype (ACS 7) was recovered in 10/64 F2 plants derived from three independent self-pollinations of F1 plants (30 ACS 1; 15 ACS 2; 2 ACS 3; 10 ACS 7). The observed frequency of *Pl-Rh*-like phenotypes (16%) is not significantly different from the 25% expected from a single locus recessive mutation ($\chi^2 = 1.95$; $P > 0.05$) but is significantly different from the 6.25% expected if the dark-anther trait is due to the presence of two unlinked recessive mutations ($\chi^2 = 9.7$; $P < 0.01$). All F2 progeny with ACS 7 anthers were 2/3 the height of their *Pl'*-like siblings. Five of the 62 F2 progeny produced barren (no flowers) tassels so it was not possible to assign these to a given Anther Color Score class. However, these five anther-less progeny were short in stature and had very strong plant color similar to all other F2 plants that had ACS 7 anthers. If we include these five anther-less progeny among the "ACS 7" mutant class, then 15/62 (24%) of the F2 progeny have the mutant phenotype. These F2 segregation ratios are consistent with the hypothesis that the dark-anther phenotype, and the dark-plant phenotype, is due to a single locus recessive mutation.

[00373] ***Mop1-5* is transmitted as a recessive mutation**. Homozygous *Mop1-5/Mop1-5* plants having a *Pl-Rh*-like phenotype were outcrossed to *Pl'/Pl'* testers. All F1 progeny plants (14 individuals from 2 independent outcrosses) had a *Pl'* anther phenotype (2 ACS 1; 9 ACS 2; 2 ACS 3; 1 ACS 4) indicating that the *Mop1-5* allele is recessive and further that *Pl'* alleles

transmitted from homozygous *Mop1-5/Mop1-5* plants are not recalcitrant to subsequent paramutation.

[00374] ***Mop1-4* and *Mop1-5* define two new alleles of *Mop1*.** Complementation crosses were made between homozygous mutant (*Pl-Rh* anthers) plants and plants heterozygous for a different mutation (*Pl'* anthers). Results (Table 15) indicate that the *Mop1-4* and *Mop1-5* mutations complement mutations at rmr*1*, rmr2, rmr6, rmr9 and rmr*1*1. However, the two mutations fail to complement each other and the *Mop1-1* mutation. If the *Mop1-4* mutation is an allele of *Mop1*, then 1/2 of all progeny (15 individuals) from the two complementation crosses with *Mop1-1* should have a *Pl-Rh*-like anther phenotype. The observed frequency of *Pl-Rh*-like types is not significantly different from the expected frequency for this hypothesis (□2 = 3.3; P > 0.05) suggesting that *Mop1-4* defines an allele of *Mop1*. If the *Mop1-5* mutation defines an allele of *Mop1*, then 50% of the complementation cross progeny made with *Mop1-1* (9-10 individuals) should have a *Pl-Rh*-like anther phenotype. The observed frequency is not significantly different from this hypothesis (□2 = 0.66; P > 0.05) suggesting that *Mop1-5* is an allele of *Mop1*.

[00375] ***Pl'* can change back to a meiotically-heritable *Pl-Rh* state in homozygous *Mop1-4* and *Mop1-5* plants.** Crosses were made to five different homozygous *Pl-Rh* tester stocks to determine if all the *pl1* alleles sexually transmitted from *Pl'/Pl'*; *Mop1-4/Mop1-4* plants remained paramutagenic as typically observed in wild type stocks (Hollick et al. 1995). Four of the 16 crosses made between *Pl-Rh/Pl-Rh* testers and *Pl'/Pl'*; *Mop1-4/Mop1-4* plants gave rise to at least one progeny plant with *Pl-Rh*-like anthers (83/278 ACS 1; 117/278 ACS 2; 61/278 ACS 3; 7/278 ACS 4; 4/278 ACS 5; 6/278 ACS 7). Crosses were made to six different homozygous *Pl-Rh* tester stocks to determine if all the *pl1* alleles sexually transmitted from *Pl'/Pl'*; *Mop1-5/Mop1-5* plants remained paramutagenic. One of the 6 crosses made between *Pl-Rh/Pl-Rh* testers and *Pl'/Pl'*; *Mop1-5/Mop1-5* plants gave rise to at least one progeny plant with *Pl-Rh*-like anthers (29/100 ACS 1; 55/100 ACS 2; 1/100 ACS 3; 7/100 ACS 4; 2/100 ACS 5; 0/100 ACS 6; 6/100 ACS 7).

[00376] Thus, preliminary data indicate that *Pl'* alleles can sometimes change back to a meiotically-heritable, non-paramutagenic or weakly-paramutagenic state in plants homozygous for *Mop1-4* and *Mop1F-5*.

[00377] **The *Mop1-4* mutation affects plant development.** The *Pl-Rh*-like phenotype seen in homozygous *Mop1-4* plants co-segregates with obvious developmental abnormalities. Homozygous *Mop1-4* plants are 1/3 shorter, on average than their heterozygous siblings. Measurements taken from three independent families in our 2000-

2001 winter nursery in Molokai, HI, show an average height of 69 inches for *Mop1-4* heterozygotes and 45 inches for homozygous siblings. In general, the *Mop1-4* homozygotes also have a slightly bushier tassel with extra branches and shorter internode distances between the branches. However, dimorphic tassel phenotypes of homozygous *Mop1-4* plants have been noted; tassels are smallish, thin, and sparsely adorned with florets in our Hawaii nurseries but relatively normal in our Albany, CA nursery. This phenotype is very similar to what has been reported for the *Mop1-1* and *Mop1-2EMS* alleles (Dorweiler et al., 2000). In all other obvious respects, *Mop1-4* homozygous plants appear to be normal.

[00378] **The *Mop1-5* mutation does not obviously affect plant development.** The *Pl-Rh*-like phenotype seen in homozygous *Mop1-5* plants is not associated with any obvious developmental abnormalities. Field observations of a single F2 family segregating the *Mop1-5* mutation as well as comparison of *Mop1-5/Mop1-5* and *Mop1-5/+* individuals derived from intercrossing *Mop1-5/Mop1-5* and *Mop1-5/+* F2 siblings show relatively uniform plant morphology.

References

[00379] The following references are cited throughout the Specification and the Examples and each is hereby incorporated by reference in their entirety:

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